Activation of Nuclear Factor κB and *bcl-x* Survival Gene Expression by Nerve Growth Factor Requires Tyrosine Phosphorylation of IκBα

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Abstract. NGF has been shown to support neuron survival by activating the transcription factor nuclear factor-κB (NFκB). We investigated the effect of NGF on the expression of Bcl-xL, an anti–apoptotic Bcl-2 family protein. Treatment of rat pheochromocytoma PC12 cells, human neuroblastoma SH-SY5Y cells, or primary rat hippocampal neurons with NGF (0.1–10 ng/ml) increased the expression of *bcl-xL* mRNA and protein. Reporter gene analysis revealed a significant increase in NFκB activity after treatment with NGF that was associated with increased nuclear translocation of the active NFκB p65 subunit. NGF-induced NFκB activity and Bcl-xL expression were inhibited in cells overexpressing the NFκB inhibitor, IκBα. Unlike tumor necrosis factor-α (TNF-α), however, NGF-induced NFκB activation occurred without significant degradation of IκBs determined by Western blot analysis and time-lapse imaging of neurons expressing green fluorescent protein–tagged IκBα. Moreover, in contrast to TNF-α, NGF failed to phosphorylate IκBα at serine residue 32, but instead caused significant tyrosine phosphorylation. Overexpression of a Y42F mutant of IκBα potently suppressed NFG-, but not TNF-α–induced NFκB activation. Conversely, overexpression of a dominant negative mutant of TNF receptor-associated factor-6 blocked TNF-α–, but not NGF-induced NFκB activation. We conclude that NGF and TNF-α induce different signaling pathways in neurons to activate NFκB and *bcl-x* gene expression.

Key words: nerve growth factor • nuclear factor-κB • Bcl-xL • tumor necrosis factor-α • IκB

Introduction

Bcl-xL is an anti–apoptotic Bcl-2 family protein that is widely expressed in the developing and adult nervous system (Boise et al., 1993; Gonzalez-Garcia et al., 1995). Target disruption of the *bcl-x* gene has demonstrated its importance for neuronal survival. *bcl-x*–deficient mice die in utero, exhibiting pronounced cell death in both the peripheral and central nervous system (Motoyama et al., 1995). *bcl-x* transcripts are alternatively spliced into long and short forms. The protein product of the long form (Bcl-xL) is a potent inhibitor of apoptosis, while the short form (Bcl-xS) accelerates apoptosis (Boise et al., 1993). Bcl-xL is the Bcl-x form predominantly expressed in neurons (Gonzalez-Garcia et al., 1995).

Little is known about the regulation of *bcl-x* gene expression in the nervous system. In blood cells, transcription of the *bcl-x* gene is controlled by transcription factors, signal transducer, and activator of transcription 5 and nuclear factor κB (NFκB)[1] (Dumon et al., 1999; Lee et al., 1999; Socolovsky et al., 1999; Chen et al., 2000). Binding sites for the active NFκB subunits p65/relA and c-rel have been demonstrated by functional analysis of the *bcl-x* promoter (Chen et al., 1999; Lee et al., 1999). Cytokines such as tumor necrosis factor (TNF)-α activate NFκB by inducing the degradation of IκB proteins. These are cytosolic proteins associated with NFκB subunits that function as their inhibitors (Baeuerle and Baltimore, 1988). Degradation of IκB proteins has been shown to involve phosphorylation at serine residues, ubiquitination, and subsequent degradation via the 26S proteasome complex (Palombella et al., 1994; Brown et al., 1995; Traenckner et al., 1995).

We have previously shown that the cytokine transforming growth factor-β1 also regulates the expression of the

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[1]*Abbreviations used in this paper:* COX-2, cyclooxygenase-2; EGFP, enhanced green fluorescent protein; NFκB, nuclear factor-κB; P-Ser32-IκBα, serine 32-phosphorylated IκBα; RT, reverse transcription; SEAP, secreted form of human placental alkaline phosphatase; TNF-α, tumor necrosis factor-α; TRAF6 dn, dominant negative tumor necrosis factor receptor-associated factor-6.

anti–apoptotic proteins Bcl-xL and Bcl-2 in primary neuron cultures (Prehn et al., 1994, 1996). Likewise, the proinflammatory cytokine TNF-α has recently been shown to increase Bcl-xL expression in neurons in an NFκB-dependent manner (Tamatani et al., 1999). However, there is growing evidence that NFκB activation is not only involved in the nervous system response to injury or inflammation, but is also required to support neuron survival during development and in the adult nervous system. Activation of excitatory amino acid receptors (Kaltschmidt et al., 1995) and release of neurotrophic factors may mediate constitutive NFκB activity in neurons (Carter et al., 1996; Maggirwar et al., 1998; Hamanoue et al., 1999; Middleton et al., 2000). NGF in particular has been shown to increase NFκB activity in various neuronal and nonneuronal populations (Wood, 1995; Carter et al., 1996; Taglialatela et al., 1997; Ladiwala et al., 1998; Maggirwar et al., 1998; Yoon et al., 1998; Hamanoue et al., 1999). The present study demonstrates that NGF regulates the expression of Bcl-xL via an NFκB-dependent pathway. Moreover, we demonstrate that NGF-induced NFκB activation requires tyrosine phosphorylation of the inhibitor IκBα, but occurs independently of serine phosphorylation and degradation of IκBs via the proteasome.

Materials and Methods

Materials

Murine 2.5S NGF and recombinant human TNF-α were from Promega. The proteasome inhibitors carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) and lactacystin were purchased from Biomol. Sodium pervanadate (Sigma-Aldrich) was prepared as described by Imbert et al. (1996). All other chemicals came in molecular biological grade purity from Promega.

Cell Culture

Rat pheochromocytoma PC12 cells were grown in DME medium (Life Technologies) supplemented with 10% horse serum (PAN Biotech), 5% FCS (PAA) and the antibiotic mixture of 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). Human neuroblastoma SH-SY5Y cells were grown in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS and the antibiotic mixture. Hippocampal neurons were prepared from neonatal (P1) 344 rats (Fisher Scientific) as described (Krohn et al., 1998). Cells were maintained in MEM supplemented with 10% NU®-Serum, 2% B-27 supplement (50× concentrate), 2 mM L-glutamine, 20 mM D-glucose, 26.2 mM sodium bicarbonate, and the antibiotic mixture (Life Technologies). Hippocampal neurons were plated onto poly-L-lysine–coated 35-mm Petri dishes (Becton Dickinson). Studies were performed on 8–10-d-old cultures. Animal care followed official governmental guidelines. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide.

Reverse Transcription PCR

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN). Purity of samples and RNA content were measured using a UV photometer (Amersham Pharmacia Biotech). 1 μg of total RNA was reverse-transcribed and amplified in a single reaction tube in a volume of 50 μl. The reverse transcription (RT) reaction was performed at 42°C for 20 min in the presence of oligo(dN) primers and Moloney murine leukemia virus reverse transcriptase (Amersham Pharmacia Biotech). After heat inactivation, specific oligonucleotide primer pairs for *bcl-x* and *GAPDH* (20 pmol each; MWG) were added to the reaction mixture (1.5 mM $MgCl_2$, 60 mM KCl, 10 mM Tris-HCl, pH 9.0, 200 μM deoxynucleotides each and 2 U Taq polymerase; Amersham Pharmacia Biotech). Primer pairs were based on *Mus musculus bcl-x* and *Rattus norvegicus GAPDH* sequences. The sequences of the primers were as follows: *bcl-x* sense primer, 5′-GGA GAG CGT TCA GTG ATC-3′ and *bcl-x* antisense primer, 5′-CAA TGG TGG CTG AAG AGA-3′; *GAPDH* sense primer, 5′-CTC GTG GTT CAC ACC CAT-3′ and *GAPDH* antisense primer, 5′-GGC TGC CTT CTC TTG TGA-3′. PCR was performed for 22 (*bcl-x*) or 15 (*GAPDH*) cycles at 94°C for 30 s, 58°C for 60 s, and 72°C for 120 s using a Primus 25 thermocycler (MWG). The amplified PCR products (expected size for *bcl-xL*: 472 bp; expected size for *GAPDH*: 355 bp) were separated on a 5% agarose gel containing 0.1% ethidium bromide and visualized using a CCD camera-based documentation system (MWG). Intensity of bands was analyzed using ONEDscan software (Scanalytics). The intensity of the *GAPDH* amplification product served as internal control. Amplification temperature and cycle number with respect to the linear range of the amplification process were empirically determined for each primer pair.

SDS-PAGE and Western Blotting

Cultures were rinsed with ice-cold PBS and lysed in TBS containing SDS, glycerin, and protease inhibitors. Protein content was determined using the BCA Micro Protein Assay kit (Pierce Chemical Co.) and samples were supplemented with 2-mercaptoethanol and denaturated at 95°C for 5 min. An equal amount of protein (20–50 μg) was separated by SDS-PAGE and blotted to nitrocellulose membranes (Protean BA 85; Schleicher & Schuell). Equal loading of samples was confirmed by Ponceau red staining. Nonspecific binding was blocked at room temperature for 2 h by incubation in TBS containing 0.1% Tween-20, BSA, and nonfat dry milk. The blots were then incubated over night at 4°C with the primary antibodies diluted in blocking buffer. Antibodies used were a rabbit polyclonal anti–Bcl-x antibody (a gift from Prof. Craig B. Thompson, University of Pennsylvania, Philadelphia, PA) diluted 1:5,000, a mouse monoclonal anti–cyclooxygenase-2 (COX-2) antibody (clone 33; Transduction Laboratories) diluted 1:2,000, a rabbit polyclonal antibody specific for serine 32-phosphorylated IκBα (P-Ser32-IκBα) (New England Biolabs, Inc.) diluted 1:2,000, rabbit polyclonal antibodies recognizing IκBα (New England Biolabs, Inc., and sc-203; Santa Cruz Biotechnology, Inc.) diluted 1:2,000, a rabbit polyclonal antibody specific for IκBβ (sc-945; Santa Cruz Biotechnology, Inc.) diluted 1:1,000, and a mouse monoclonal anti–α-tubulin antibody (clone DM 1a; Sigma-Aldrich) diluted 1:2,000. Afterwards, membranes were washed and incubated with anti–mouse or –rabbit IgG-HRP conjugate (1:5,000; Promega). Antibody-conjugated peroxidase activity was visualized using the Super Signal chemiluminescence reagent (Pierce Chemical Co.). Immunoblots were stripped in stripping buffer (2% SDS, 62.5 mM Tris-HCl, 100 mM 2-mercaptoethanol, pH 6.8) for 20 min at 60°C, washed, and reprobed.

Immunocytochemistry and Visualization of the Active p65 NFκB Subunit

After stimulation with NGF, cells were washed and fixed with 4% paraformaldehyde in PBS at 37°C for 20 min. Fixed cells were permeabilized by treatment with ice-cold 0.1% Triton X-100 in PBS. Nonspecific antibody binding was blocked by PBS, pH 7.4, containing 2% nonfat dry milk, 2% BSA, and 0.1% Tween 20 for 1 h at room temperature. The active p65 NFκB subunit was visualized using a mouse monoclonal antibody (clone 12H11; Roche) that recognizes an epitope overlapping the nuclear location signal of the p65 NFκB subunit (Zabel et al., 1993; Kaltschmidt et al., 1995). The antibody was diluted 1:100 in blocking buffer and incubated overnight at 4°C. Afterwards, cells were washed with PBS and labeled with biotin-conjugated anti–mouse IgG (1:1,000; Vector Laboratories) for 1 h at room temperature. After being washed, cells were incubated for 30 min in a mixture of avidin and biotinylated HRP reagent (Vectastain Elite ABC Kit; Vector Laboratories). Staining procedure was performed using DAB as chromogen (1 mg/ml) in the presence of Ni^{2+} and hydrogen peroxide. After 8–10 min, the chromogen was washed out and cells were observed in transmitted light using an Eclipse TE300 inverted microscope (Nikon). Digital images of equal exposure were acquired with a SPOT-2 camera (Diagnostic Instruments).

Analysis of NFκB Reporter Gene Activity

PC12 cells were seeded on poly-L-lysine coated 24-well plates at a density of 10^4 cells per well. Cells were then transfected with 0.75 μg of a plasmid containing four tandem repeats of the κ enhancer element fused to the herpes simplex virus thymidine kinase promoter upstream of the coding sequence for a secreted form of human placental alkaline phosphatase (SEAP) (pNFκB-SEAP; CLONTECH Laboratories, Inc.). In the experiment shown in Fig. 3 (below), PC12 cultures were cotransfected with 0.25 μg of a human wild-type IκBα expression plasmid (pIκBα) controlled by the cytomegalovirus promoter or control DNA of similar kilobase size. The IκBα expression plasmid was originally generated and published by Brockman et al. (1995). In the experiment shown in Fig. 5 (below), PC12 cells

were cotransfected with 0.05 μg of a wild-type IκBα or mutant IκBα Y42F pcDNA expression plasmid (Imbert et al., 1996). In the experiments shown in Fig. 6 (below), PC12 cells were cotransfected with 0.01 or 0.05 μg of a dominant-negative TNF receptor-associated factor-6 (TRAF6 dn) expression plasmid (a gift from Dr. H. Wajant, University of Stuttgart, Stuttgart, Germany). For the generation of the dominant-negative mutant, a cDNA fragment comprising human TRAF6 (253–522) with a 5′ BamHI site and a 3′ NotI site was cloned into expression vector pcDNA3.1 (Invitrogen). Cells were transfected using polyethylenimine or the Lipofectamine transfection reagent (Life Technologies). 24–48 h after transfection, basal SEAP in the culture medium was washed out and fresh media containing NGF, TNF-α, or vehicle was added. The medium was collected after 6 and 24 h and a fluorescence SEAP assay was performed (Great EscaAPe Fluorescence Detection Kit; CLONTECH Laboratories, Inc.) using 4-methylumbelliferyl phosphate as substrate for SEAP. Fluorescence intensity was measured using a fluorescence plate reader (HTSoft 7000; PerkinElmer) (360 nm excitation, 465 nm emission). Each set of experiments included cultures transfected with a plasmid identical to the reporter construct but lacking the κ enhancer element as negative control for background signals. As control for transfection efficiency, cultures were cotransfected with a pSV–β-galactosidase plasmid, and NFκB reporter gene activity was normalized to β-galactosidase expression.

Overexpression of IκBα and Immunofluorescence Analysis

PC12 cells were plated onto poly-L-lysine–coated eight-well tissue culture slides (Becton Dickinson) at a density of 10^4 cells per well. Cultures were then cotransfected with 0.25 μg of pIκBα or control plasmid DNA, as well as a plasmid encoding enhanced green fluorescent protein (EGFP) as transfection control. After 72 h recovery, cells were exposed to NGF, TNF-α, or vehicle for 6 h, fixed, and permeabilized as described above. After blocking for 1 h at room temperature, the specimens were incubated at 4°C overnight with the rabbit polyclonal Bcl-x antibody diluted 1:500 in blocking buffer. After washing, biotin-conjugated anti–rabbit IgG (1:1,000; Vector Laboratories) was added for 1 h at room temperature, followed by a streptavidin–Texas red conjugate (1 μg/ml, 20 min; Molecular Probes). Texas red fluorescence was observed using the Eclipse TE300 inverted microscope and a 40× oil immersion objective (Nikon) with the following optics: 510–560 nm excitation, 575 nm dichroic mirror, >590 nm emission. For the observation of the EGFP fluorescence, the following optics were used: 465–495 nm excitation, 505 nm dichroic mirror, 515–555 nm emission. Digital images of equal exposure were acquired with the SPOT-2 camera using Metamorph software (Universal Imaging Corp.). For quantification of Bcl-xL immunoreactivity, average pixel intensity of the Texas red fluorescence of single cells expressing EGFP was measured using Metamorph software. Background fluorescence intensities of the specimen were substracted from the values.

Immunoprecipitation

After exposure to NGF, sodium pervanadate, or vehicle, PC12 cells were rinsed with PBS and lysed in buffer containing (mM): 50 Tris-HCl, pH 7.5, 0.5% NP-40, 10% glycerol, 250 NaCl, 5 EDTA, 50 NaF, 0.5 Na_3VO_4, 10 β-glycerophosphate, and the protease inhibitors PMSF (0.5 mM), leupeptin, and aprotinin (5 μg/ml). Protein content was determined and 250 μg protein extract was immunoprecipitated using a mouse monoclonal antibody (0.5 μg) recognizing tyrosine-phosphorylated proteins (sc-508; Santa Cruz Biotechnology, Inc.). As negative control, lysates were immunoprecipitated using mouse control IgG (Santa Cruz Biotechnology, Inc.). Samples were rotated overnight at 4°C and the antibody–protein complexes were precipitated for 2 h at 4°C using protein agarose A/G plus (Santa Cruz Biotechnology, Inc.). The beads were centrifuged and washed four times. Samples were supplemented with 2-mercaptoethanol and denatured at 95°C for 2 min. Immunoprecipitated proteins were subjected to 10% SDS-PAGE, blotted, and detected using a rabbit polyclonal anti–IκBα antibody (sc-203; Santa Cruz Biotechnology, Inc.), diluted 1:2,000. For immunoprecipitation control, the supernatants were collected and aliquots were subjected to SDS-PAGE. IκBα protein was not detectable in the supernatants.

Time-Lapse Imaging of Neurons Expressing IκBα-EGFP

PC12 cells were plated at a density of 10^4 cells on 35-mm glass-bottom dishes (Willco BV) coated with poly-L-lysine. Cultures were then transfected with 0.75 μg of a plasmid encoding an IκBα-EGFP fusion protein (pIκBα-EGFP; CLONTECH Laboratories, Inc.) or a plasmid expressing EGFP. After 24-h recovery, EGFP fluorescence was observed using an Eclipse TE 300 inverted microscope and a 40× oil immersion objective equipped with the appropriate filter set (465–495 nm excitation, 505 nm dichroic mirror, 515–555 nm emission). Time-lapse digital images of equal exposure were acquired with the SPOT-2 camera using Spot software version 2.2.1. After acquiring the first image, cells transfected with pIκBα-EGFP were incubated with NGF, TNF-α, or vehicle directly on the stage. In control experiments, cultures transfected with EGFP were exposed to TNF-α. The incubation medium was enriched with 10 mM Hepes and thoroughly mixed to ensure a proper distribution of the agents. Images were analyzed using Metamorph software. Fluorescence data are given as change in average pixel intensity compared with the first image. Background fluorescence of each image was subtracted from the values.

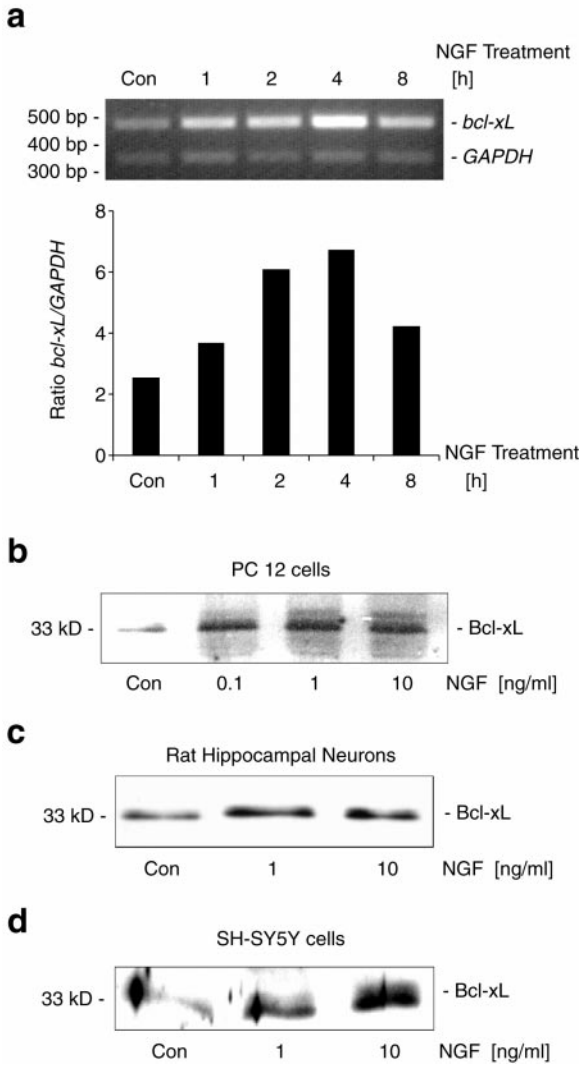

Figure 1. NGF increases neuronal Bcl-xL expression. (a, Top) Rat pheochromocytoma PC12 cells were treated with NGF (10 ng/ml) or vehicle (Con) for the indicated period of time and expression of *bcl-xL* and *GAPDH* (internal control) mRNA was determined by RT-PCR. (Bottom) Intensity of *bcl-xL* bands relative to *GAPDH* plotted against the respective time points from the same experiment. The experiment was performed twice with similar results. Rat PC12 cells (b), rat hippocampal neuron cultures (c) and human neuroblastoma SH-SY5Y cells (d) were exposed to NGF or vehicle. After 6 h, cytosolic protein extracts were subjected to 15% SDS-PAGE. Immunodetection of Bcl-xL was performed using a rabbit polyclonal antibody specific for Bcl-x. Experiments were performed in duplicate or triplicate with comparable results.

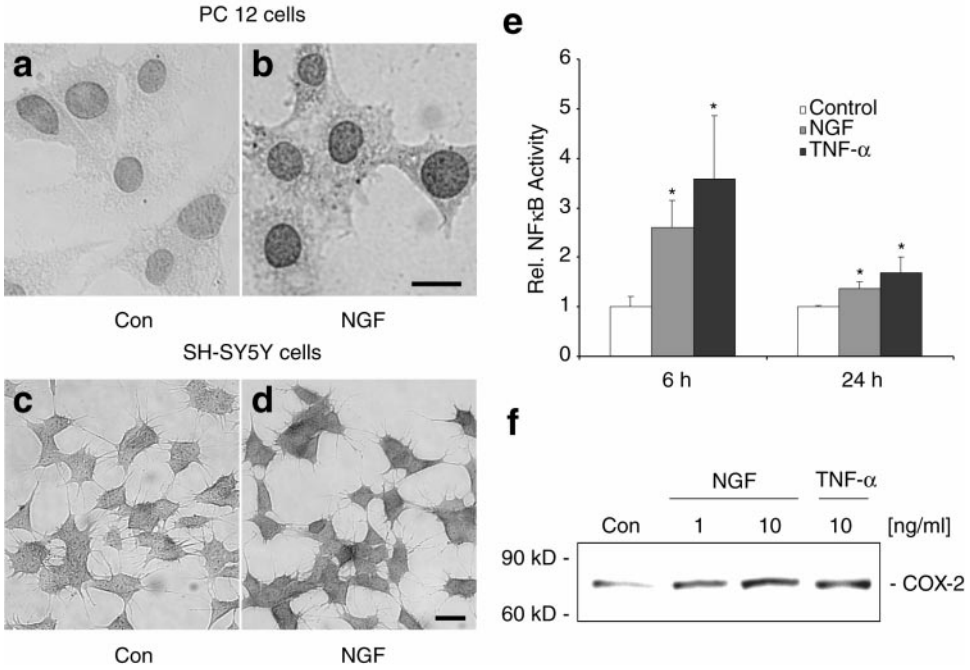

Figure 2. NGF activates NFκB. Immunocytochemical detection of the active p65 subunit. Rat pheochromocytoma PC12 cells were treated with vehicle (a) or 1 ng/ml NGF (b) for 4 h, fixed, and probed with a mouse monoclonal antibody that recognizes an epitope overlapping the nuclear translocation signal of the p65 NFκB subunit. Note the increased active p65 immunoreactivity in the nuclei. Human neuroblastoma SH-SY5Y cells were treated with vehicle (c) or 10 ng/ml NGF (d) for 4 h and analyzed as described above. Note the increased active p65 immunoreactivity in the nuclei and the cytoplasm. Scale bar, 25 μm. (e) NF-κB activity determined in PC12 cells by reporter gene assay. Cells were transfected with a reporter plasmid (pNFκB-SEAP). 24 h after transfection, cultures were treated with NGF (1 ng/ml), TNF-α (10 ng/ml), or vehicle. Culture medium was collected after 6 and 24 h of treatment and a fluorescent SEAP assay was performed. Data are means ± SEM from $n = 14$–15 cultures in three separate experiments. (f) Western blot analysis of the NFκB-regulated gene product COX-2 in PC12 cells treated for 6 h with vehicle, NGF, or TNF-α.

Statistics

Data are presented as means ± SEM. For statistical comparison, one-way analysis of variance followed by LSD test were employed. Kruskal-Wallis H test followed by Bonferroni-corrected Mann-Whitney U test were used for statistical evaluation of nonparametric data. $P < 0.05$ was considered to be statistically significant.

Results

Nerve Growth Factor Upregulates Neuronal bcl-xL mRNA and Bcl-xL Protein Expression

To investigate the effect of NGF on neuronal *bcl-xL* mRNA expression, PC12 cells were treated with 10 ng/ml NGF for time periods of 1–8 h. Total RNA was isolated and subjected to semiquantitative RT-PCR using *bcl-x* and *GAPDH*-specific oligonucleotide primers. *bcl-xL* mRNA expression increased in the PC12 cells after 2 h of NGF treatment, remained at a high level after 4 h, and then declined after 8 h (Fig. 1 a). In agreement with the RT-PCR data, we detected increased Bcl-xL protein expression in PC12 cells treated with NGF (Fig. 1 b). Interestingly, a strong response was already observed in cultures treated with NGF concentrations as low as 0.1 and 1 ng/ml. The Bcl-xL level of cultures treated with very high NGF concentrations (50–100 ng/ml) declined again (data not shown).

NGF also increased Bcl-xL protein expression in primary rat hippocampal neurons, with maximal effects again seen after treatment with 1 ng/ml NGF (Fig. 1 c). *bcl-xL* mRNA and Bcl-xL protein expression was also increased in human neuroblastoma SH-SY5Y cells treated with NGF (Fig. 1 d and data not shown).

Nerve Growth Factor Activates NFκB

NFκB binding sequences have been identified in the promoter region of the human and murine *bcl-x* gene (Chen et al., 1999; Lee et al., 1999). To obtain evidence for increased NFκB activity after treatment with NGF, we performed an immunocytochemical analysis in PC12 and SH-SY5Y cells using a monoclonal antibody raised against an epitope overlapping the nuclear localization signal of the NFκB p65 subunit. This epitope is normally inaccessible because of binding of the endogenous inhibitor IκB (Zabel et al., 1993; Kaltschmidt et al., 1995). Dissociation of the inhibitor exposes the nuclear localization signal of p65, enabling it to be imported into the nucleus and to activate transcription (Baeuerle and Baltimore, 1988). Treatment of PC12 cells with NGF induced a strong increase in the immunoreactivity of the active NFκB subunit p65 in the nucleus with maximal effects observed at a concentration of 1 ng/ml (Fig. 2, a and b). Treatment of human SH-SY5Y neuroblastoma cells with NGF also increased the immunoreactivity of the active p65 subunit, both in the cytoplasm and in the nucleus (Fig. 2, c and d).

To provide direct evidence for increased NFκB activity after NGF treatment, PC12 cells were transfected with a reporter plasmid containing four tandem repeats of the κ enhancer element fused to the herpes simplex virus thymidine kinase promoter upstream of the coding sequence for the reporter gene SEAP. Treatment of PC12 cells with 1 ng/ml NGF induced NFκB activity to an extent comparable with that induced by TNF-α (10 ng/ml), a cytokine that is known to increase NFκB activity in neurons (Barger et al., 1995) and that served as a positive control (Fig. 2 e). Increased NFκB activity could also be detected by re-

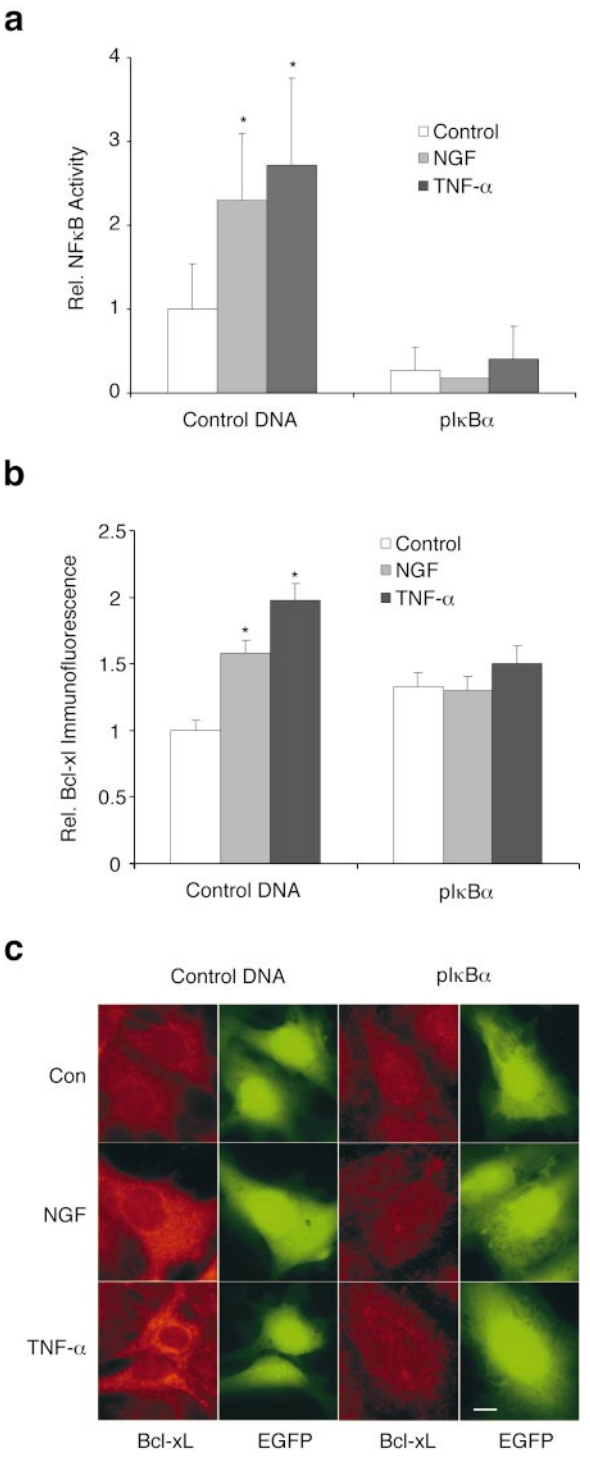

Figure 3. IκBα overexpression inhibits NGF-induced NFκB activity and Bcl-xL expression. (a) PC12 cells were transiently cotransfected with a IκBα expression vector or control plasmid DNA (control DNA) and the reporter plasmid pNFκB-SEAP. 48 h after transfection, cultures were treated with NGF (1 ng/ml), TNF-α (10 ng/ml), or vehicle. Culture media were collected after 6 h and a fluorescence SEAP assay was performed. Data are mean ± SEM from n = 5–6 cultures per treatment. A duplicate experiment yielded comparable results. (b and c) PC12 cells were cotransfected with pCMV-IκBα or control plasmid and pCMV-EGFP as transfection control. After a 72-h recovery, cultures were exposed to NGF (1 ng/ml), TNF-α (10 ng/ml), or vehicle for 6 h and fixed. Bcl-xL expression was determined by immunofluorescence analysis using the Bcl-x antibody followed by biotinylated secondary antibody and streptavidin Texas red conjugate. Bcl-xL immunofluorescence was quantified by measuring the average pixel intensity of the Texas red fluorescence of single cells expressing EGFP. Data are mean ± SEM from n = 17–70 cells in two to three separate cultures per treatment. Experiments were repeated two times with similar results. Scale bar, 10 μm.

porter gene analysis in human SH-SY5Y neuroblastoma cells exposed to NGF (data not shown).

Finally, evidence for increased NFκB activity after NGF treatment was provided by determining the expression of the NFκB target gene COX-2 (Yamamoto et al., 1995). Expression of COX-2 protein increased after treatment with NGF to a similar extent as after treatment with TNF-α (Fig. 2 f).

NGF-Induced NFκB Activity and Bcl-xL Expression Is Inhibited in PC12 Cells Overexpressing IκBα

To investigate the requirement of NFκB activation for NGF-induced Bcl-xL expression, PC12 cells were transiently transfected with a plasmid encoding the NFκB inhibitor IκBα or control DNA. Overexpression of IκBα reduced both NGF- and TNF-α–induced NFκB activity analyzed by the reporter gene assay (Fig. 3 a). We next analyzed Bcl-xL protein expression in IκBα-overexpressing PC12 cells cotransfected with EGFP as transfection control. In agreement with our observations described above (Fig. 1), Bcl-xL immunofluorescence increased significantly after a 6-h treatment with NGF (1 ng/ml) in cultures transfected with control DNA (Fig. 3, b and c). Increased Bcl-xL expression was also observed in control-transfected PC12 cells exposed to TNF-α (10 ng/ml). Overexpression of IκBα did not significantly alter the level of Bcl-xL expression in vehicle-treated control cells (ANOVA and LSD test; P = 0.131). Interestingly, however, NGF and TNF-α failed to increase neuronal Bcl-xL expression in cells overexpressing the inhibitor.

TNF-α, but Not NGF Induces Rapid Degradation of IκBs

IκB degradation has been shown to be required for activation of NFκB by proinflammatory cytokines (Palombella et al., 1994). We therefore determined a time course of IκBα protein degradation in PC12 cells exposed to TNF-α or NGF (Fig. 4, a and b). TNF-α induced significant IκBα degradation, starting 10 min after the onset of treatment. In contrast, treatment with NGF for up to 8 h failed to induce significant degradation of IκBα. NGF also failed to trigger the degradation of a second NFκB inhibitory protein, IκBβ (Thompson et al., 1995). In contrast, IκBβ degradation also occurred in TNF-α–treated cultures, albeit with slower kinetics.

To analyze IκBα degradation in response to NGF and TNF-α on the single cell level, PC12 cells were transfected with a plasmid encoding EGFP-tagged IκBα. The fusion protein has been previously shown to be degraded after serine phosphorylation with kinetics similar to IκBα, resulting in a decrease in cellular IκBα-EGFP fluorescence (Li et al., 1999). Treatment of IκBα-EGFP–transfected

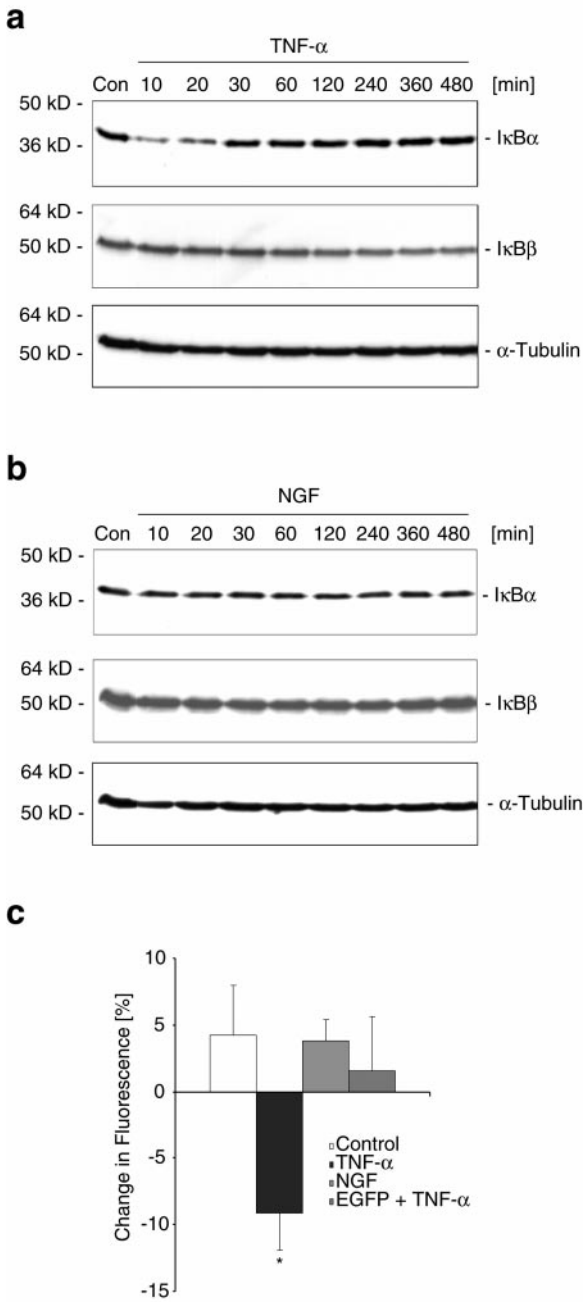

Figure 4. TNF-α, but not NGF induces degradation of IκBs α and β. Degradation of IκBα and IκBβ in PC12 cells treated with (a) TNF-α (10 ng/ml) or (b) NGF (1 ng/ml) for the indicated period of time. 50 μg protein extract were separated on 12% SDS-PAGE, blotted onto nitrocellulose membrane, and IκBα or β was detected using rabbit polyclonal antibodies. Membranes were stripped and probed with an α-tubulin mouse monoclonal antibody as control for equal sample loading. Experiments were performed in triplicate with similar results. (c) PC12 cells were transiently transfected with plasmids encoding an IκBα-EGFP fusion protein or EGFP. After 24–48 h recovery, cells were treated with vehicle, NGF (1 ng/ml), or TNF-α (10 ng/ml). Cells overexpressing EGFP were exposed to TNF-α (EGFP + TNF-α). Quantification of changes in EGFP fluorescence after a 10-min exposure to vehicle, NGF, or TNF-α. Data are mean ± SEM from $n = 4$ separate transfection experiments per treatment. Data are given as change in average pixel intensities compared with the first image.

cells with TNF-α induced a significant decrease in fluorescence after 10 min of exposure (Fig. 4 c). In contrast, cellular IκBα-EGFP fluorescence did not decrease in cells treated with NGF or vehicle monitored up to 2 h. As a control for specificity of the TNF-α induced decrease in IκBα-EGFP fluorescence, PC12 cells were transfected with a plasmid encoding EGFP. Treatment with TNF-α did not induce a decrease in cellular EGFP fluorescence.

IκBα Is Not Phosphorylated at Serine 32 after NGF Stimulation

IκBα has been shown to be phosphorylated at serine 32 and 36 residues after treatment with NFκB-inducing cytokines (Brown et al., 1995; Traenckner et al., 1995). To investigate serine phosphorylation of IκBα after treatment with NGF, we performed immunoblot experiments using an antibody specific for P-Ser32-IκBα. While treatment of PC12 cells with TNF-α induced an increase in P-Ser32-IκBα after 10 min of exposure with maximal effects seen after 30 min, NGF failed to induce any significant increase in P-Ser32-IκBα up to 8 h after its addition to the cultures (Fig. 5 a). At this time point, NGF had already caused a pronounced increase in NFκB activity (Fig. 2). The (lack of) effect was independent of the NGF concentration used, as similar results were obtained in cultures treated with 10 ng/ml NGF (data not shown). TNF-α–, but not NGF-induced serine 32 phosphorylation of IκBα was also observed in human SH-SY5Y neuroblastoma cells (Fig. 5 b).

Tyrosine Phosphorylation of IκBα in NGF-stimulated PC12 Cells

Phosphorylation of IκBα at tyrosine residue 42 has been shown to activate NFκB without requiring IκBα degradation via the proteasome (Imbert et al., 1996; Béraud et al., 1999). We performed immunoprecipitation experiments to analyze tyrosine phosphorylation of IκBα. PC12 cells were exposed to NGF (10 ng/ml) or sodium pervanadate (200 μM) as a positive control, and cytosolic extracts were subjected to immunoprecipitation using a murine monoclonal antibody raised against tyrosine-phosphorylated proteins. As a negative control, cell lysates were immunoprecipitated with mouse control IgG. Detection of immunoprecipitated proteins by SDS-PAGE and Western blot analysis using an IκBα antibody demonstrated that sodium pervanadate induced significant tyrosine phosphorylation of IκBα (Fig. 5 c). Treatment with NGF also induced strong tyrosine phosphorylation of IκBα after 120 min of treatment. Tyrosine-phosphorylated IκBα could also be detected in response to TNF-α (data not shown), presumably a consequence of the known ability of NFκB-activating cytokines to stimulate NGF synthesis in neural cells (Hattori et al., 1993; Friedman et al., 1996).

To investigate whether phosphorylation of IκBα at tyrosine residue 42 mediated NGF-induced NFκB activation, cells were transfected with a plasmid encoding wild-type IκBα or a mutant IκBα (Y42F), which has been shown to block NFκB activation after tyrosine phosphorylation of IκBα (Imbert et al., 1996). We titrated down the amount of wild-type IκBα plasmid DNA to a concentration at which NGF and TNF-α were still able to elicit significant NFκB activation (Fig. 5 d).

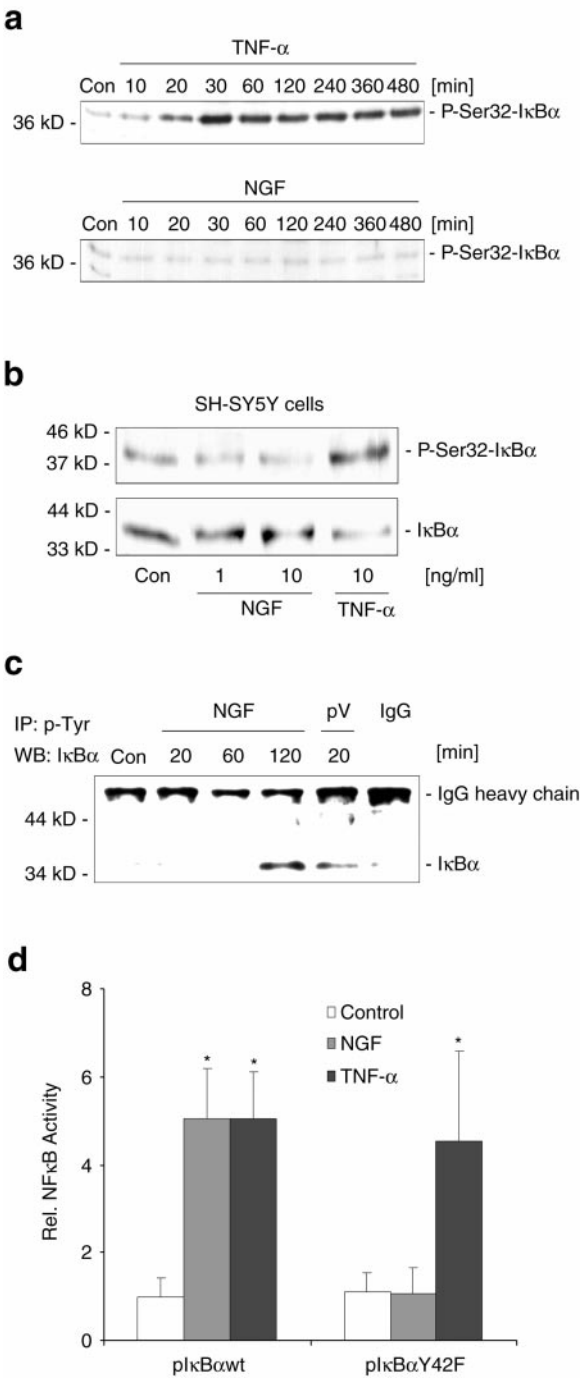

Figure 5. NGF induces tyrosine phosphorylation of IκBα. (a) PC12 cells were exposed to TNF-α (10 ng/ml) or NGF (1 ng/ml) for the indicated period of time. An equal amount of protein was subjected to 12% SDS-PAGE and blotted onto nitrocellulose membrane. Immunodetection was performed using a rabbit polyclonal antibody specific for P-Ser32-IκBα. Protein samples were identical to those shown in Fig. 4, a and b. The experiment was performed in triplicate with similar results. (b) SH-SY5Y cells were stimulated with the indicated concentrations of NGF, TNF-α, or vehicle for 6 h. Cells were collected and lysed, and total protein amount was determined. After separation on 12.5% SDS-PAGE, the P-Ser32-IκBα antibody was used to detect serine 32-phosphorylation (top). Afterwards, membranes were stripped and probed with a rabbit polyclonal antibodies recognizing IκBα (bottom). (c) PC12 cells were stimulated with NGF (10 ng/ml), sodium pervanadate (pV; 200 μM), or vehicle for the indicated period of time. Cells were lysed and 250 μg protein was immunoprecipitated using a mouse monoclonal antibody raised against tyrosine phosphorylated proteins (p-Tyr) or mouse control IgG (IgG). After precipitation and denaturation of the antibody–protein complex, samples were subjected to 10% SDS-PAGE and blotted. Immunodetection was performed using a rabbit polyclonal antibody specific for IκBα. Experiments were performed three times with similar results. (d) Effect of overexpression of wild-type and Y42F-mutated IκBα on NGF-induced NFκB activation. PC12 cells were cotransfected with the IκBα-encoding plasmids and the reporter plasmid pNFκB-SEAP. 24 h after transfection, cells were stimulated with NGF (1 ng/ml), TNF-α (10 ng/ml), or vehicle. Culture medium was collected after 6 h and a fluorescence SEAP assay was performed. Data are mean ± SEM from n = 18 cultures in three separate experiments.

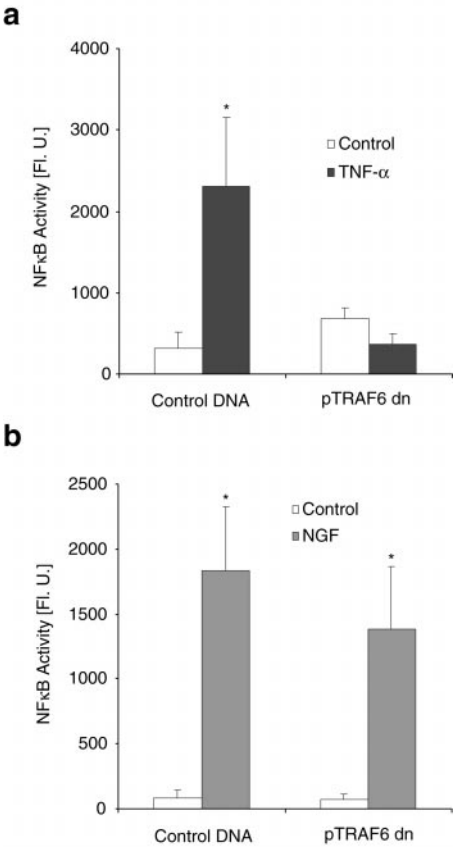

Figure 6. Overexpression of dominant-negative TRAF-6 inhibits TNF-α, but not NGF-induced NFκB activation. PC12 cells were cotransfected with (a) 0.01 or (b) 0.05 μg of a TRAF6 dn expression vector or control plasmid DNA (control DNA) and the reporter construct pNFκB-SEAP. 24 h after transfection, cells were stimulated with (a) TNF-α (10 ng/ml), (b) NGF (1 ng/ml), or vehicle for 6 h. Culture media were collected and a fluorescence SEAP assay was performed. Data are mean ± SEM from n = 5–6 cultures per treatment.

Transfection with the same concentration of plasmid DNA encoding mutant Y42F IκBα led to a complete inhibition of NGF-induced NFκB activation. In contrast, TNF-α–induced NFκB activation was not inhibited by the IκBα Y42F mutant.

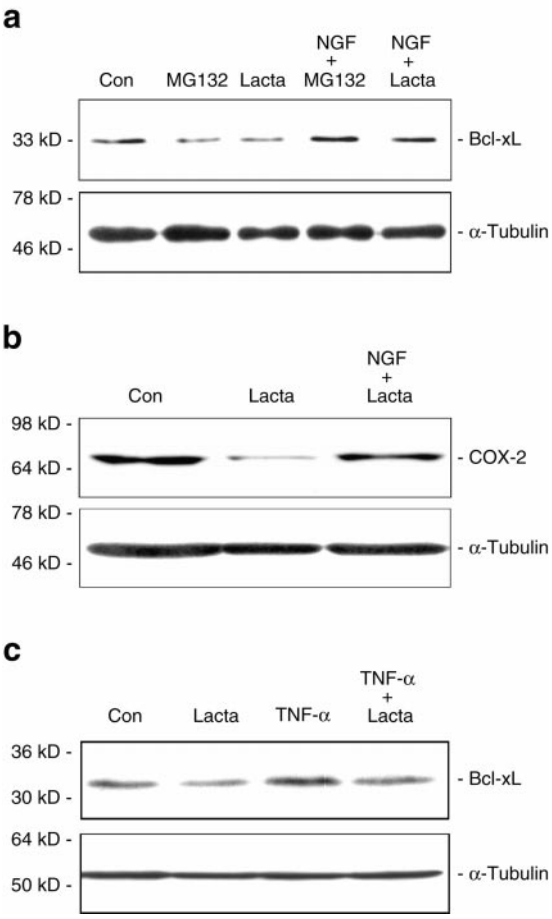

Figure 7. Proteasome inhibition and NGF-induced Bcl-xL expression. (a) PC12 cells were pretreated with MG132 (10 μM), lactacystin (Lacta; 20 μM), or vehicle for 30 min followed by a 6-h stimulation with NGF (10 ng/ml) or vehicle. Cells were lysed and 30 μg protein was subjected to 15% SDS-PAGE. Separated proteins were blotted onto nitrocellulose membrane and Bcl-xL expression was immunodetected using the α- Bcl-x antibody (top). (b) Immunodetection of the NFκB-inducible gene product COX-2 after pretreatment with lactacystin (Lacta; 20 μM) or vehicle for 30 min followed by a treatment with NGF (10 ng/ml) for 6 h (top). (c) Bcl-xL protein expression in PC12 cells after pretreatment with lactacystin (Lacta; 20 μM) or vehicle for 30 min followed by a stimulation with TNF-α (10 ng/ml) or vehicle for 6 h (top). α-Tubulin mouse monoclonal antibody served as control for detection of equal sample loading after stripping of membranes (bottom).

Overexpression of a Dominant-Negative Mutant of TRAF-6 Inhibits TNF-α-, but Not NGF-induced NFκB Activation

TRAF proteins are proximal signaling components required for TNF-α–induced NFκB activation (Rothe et al., 1995). In a similar pathway, selective activation of NFκB via p75 NGF receptors has been shown to involve the association of TRAF6 to the receptor complex (Khursigara et al., 1999; Ye et al., 1999; Foehr et al., 2000). To demonstrate differential activation pathways for TNF-α and NGF upstream of IκB phosphorylation, we transiently expressed TRAF6 dn in PC12 cells (Fig. 6). Overexpression of TRAF6 dn potently inhibited TNF-α–, but failed to inhibit NGF-induced NFκB activation.

NGF Induces Bcl-xL Expression in the Presence of Proteasome Inhibitors

NFκB activation via tyrosine phosphorylation of IκBα occurs independently of IκBα ubiquitination and degradation via the proteasome (Imbert et al., 1996; Béraud et al., 1999). To demonstrate that NGF was able to induce the expression of NFκB-target genes independent of the proteasome, we treated PC12 cells with two proteasome inhibitors, MG132 and lactacystin. Treatment with these inhibitors significantly reduced basal Bcl-xL expression in PC12 cells (Fig. 7 a). As expected, treatment with NGF was able to induce a strong increase in Bcl-xL expression in the presence of the two inhibitors. Semiquantitative RT-PCR revealed that NGF also increased neuronal *bcl-xL* mRNA expression in cultures treated with proteasome inhibitors (data not shown). Similarly, NGF increased the expression of the NFκB target gene COX-2 in the presence of the proteasome inhibitor lactacystin (Fig. 7 b). In contrast, treatment with lactacystin inhibited TNF-α–induced Bcl-xL expression (Fig. 7 c).

Discussion

In the present study, we have demonstrated that NGF induced the expression of the antiapoptotic protein Bcl-xL in rat pheochromocytoma PC12 cells, human neuroblastoma SH-SY5Y cells, and primary rat hippocampal neurons. Upregulation of *bcl-x* expression in response to NGF has previously been observed in PC12 cells (Rong et al., 1999; Foehr et al., 2000). Bcl-xL is required for the survival of many peripheral and central neurons during development, and has been shown to protect cells against a variety of metabolic and toxic challenges such as trophic factor withdrawal, oxidative stress, and hypoxia/ischemia (Boise et al., 1993; Gonzalez-Garcia et al., 1995; Parsadanian et al., 1998; Wiessner et al., 1999). NGF-induced Bcl-xL expression was inhibited in cells overexpressing the NFκB inhibitor IκBα, indicating that NFκB activation was required for the upregulation of neuronal Bcl-xL expression (Tamatani et al., 1999; Foehr et al., 2000). NGF also increases the expression of Bcl-2 (Riccio et al., 1999), an antiapoptotic protein closely related to Bcl-xL. Interestingly, upregulation of Bcl-2 by NGF involves a CREB-dependent transcriptional pathway (Liu et al., 1999; Riccio et al., 1999). It is remarkable that NGF has the capacity to increase the neuronal expression of two antiapoptotic proteins with a similar mechanism of action, via two separate transcriptional pathways. Activation of NFκB has also been shown to increase the expression of inhibitor of apoptosis proteins (You et al., 1997; Stehlik et al., 1998). These proteins have been identified as NGF-inducible target genes in chicken (Wiese et al., 1999), and function as endogenous inhibitors of a family of proapoptotic cysteine proteases, the caspases. It is therefore conceivable that the survival-promoting effects of NGF are largely mediated via an increased transcription of genes that are conserved components of the apoptotic cell death machinery.

TNFs-α and -β, as well as cytokines of the interleukin-6 family have also been shown to increase NFκB activity in

cultured neurons (Barger et al., 1995; Middleton et al., 2000). In the present study, treatment with NGF induced a significant NFκB activity comparable with that induced by TNF-α. Of note, our data demonstrate that the pathway activated by NGF is distinct from that activated by TNF-α. NFκB activity induced by TNF-α involves serine phosphorylation of IκB proteins via IκB kinases (DiDonato et al., 1997; Malinin et al., 1997; Mercurio et al., 1997), resulting in the subsequent ubiquitination and degradation by the proteasome. Both events, serine phosphorylation of IκBα and degradation of IκB proteins, could be clearly detected in response to TNF-α. In contrast, NGF did not lead to significant serine phosphorylation, but instead induced tyrosine phosphorylation of IκBα. The effect of NGF was mimicked by pervanadate, an agent that activates NF-κB via tyrosine phosphorylation of IκBα at residue 42 (Imbert et al., 1996; Singh et al., 1996). Importantly, overexpression of a Y42F mutant of IκBα potently suppressed NFG-, but not TNF-α–induced NFκB activation. This suggests that (a) tyrosine phosphorylation at this site is required for NGF-induced NFκB activation, and (b) TNF-α–induced serine phosphorylation of IκBα is sufficient to activate NFκB.

Phosphorylation of IκBα on tyrosine residue 42 has also been observed pathophysiologically in response to hypoxia/reoxygenation (Koong et al., 1994). However, our report is the first demonstration of ligand-induced tyrosine phosphorylation of IκBα. Tyrosine-phosphorylated IκBα has been reported to have a half life similar to that of non-phosphorylated IκBα, and activation of NFκB may occur by a degradation-independent dissociation of the inhibitor from the p65 subunit (Imbert et al., 1996; Béraud et al., 1999). Cultures treated with NGF indeed failed to provide strong evidence for IκBα degradation. We cannot fully exclude the absence of IκBα degradation in light of the rapid turnover of IκBα in cultured neurons and the fact that IκBα is rapidly resynthesized as a consequence of NFκB activation (Maggirwar et al., 1998). Interestingly, however, NGF induced Bcl-xL expression in the presence of proteasome inhibitors, suggesting that serine phosphorylation, ubiquitination, and degradation via the proteasome were in fact not involved in NGF-induced NFκB activation. NGF activates the PI3-kinase/Akt kinase pathway in neurons (Yao and Cooper, 1995; Crowder and Freeman, 1998; Xue et al., 2000). Béraud et al. (1999) have recently demonstrated that both the regulatory p85 and the catalytic p110 subunit of PI3-kinase are involved in NFκB activation after tyrosine phosphorylation of IκBα. The authors observed an interaction of the COOH-terminal SH2 domain of p85 with tyrosine phosphorylated IκBα. It remains to be shown whether this interaction induces a dissociation of the IκBα/NFκB complex in the absence of IκBα degradation. However, NGF-induced PI3-kinase/Akt activation may also stimulate additional regulatory steps in NFκB-dependent gene transcription; for example, by increasing the transactivation potential of NFκB (Madrid et al., 2000).

The rat pheochromocytoma PC12 and the human neuroblastoma SH-SY5Y cells used in the present study express both the trkA and p75 neurotrophin receptors (our unpublished data). Submaximal NGF concentrations (0.1–1 ng/ml) were sufficient to induce NFκB activity and Bcl-xL expression, suggesting that the p75 receptor may play a role in potentiating the effect of NGF transduced via the trkA receptor (Davies et al., 1993). Interestingly, the p75 receptor has also been shown to signal NGF-induced NF-κB activity in the absence of trkA receptors (Carter et al., 1996; Ladiwala et al., 1998; Yoon et al., 1998). Moreover, NGF-induced NFκB activation via selective activation of p75 in the trkA-deficient Schwann cell line RN22 has been shown to involve both serine phosphorylation and degradation of IκBα (Gentry et al., 2000). It is conceivable that, in the absence of trkA, NGF activates NF-κB via a pathway resembling that induced by TNF-α. In support of this concept, the p75 receptor also associates with TRAF proteins (Khursigara et al., 1999; Ye et al., 1999), adaptor proteins that are required for TNF-α–induced NFκB activation (Rothe et al., 1995). In the present study, TNF-α–induced NFκB activation in PC12 cells was potently inhibited by overexpression of a dominant-negative mutant of TRAF6, while NGF-induced NFκB activation was not suppressed. Because selective activation of NFκB via p75 NGF receptors has been shown to be sensitive to overexpression of dominant-negative TRAF6 (Khursigara et al., 1999; Foehr et al., 2000), NGF-induced NFκB activation in PC12 cells primarily involved a signal transduction pathway downstream of trkA receptors. Nevertheless, the extent of serine and tyrosine phosphorylation of IκBs as well as the extent of IκB degradation via the proteasome in response to NGF may vary in different cell types depending on the relative contribution of the signal transduction pathways activated by p75 and trkA receptors (Hamanoue et al., 1999).

There is also evidence for a cross talk between Akt and IκB kinase activation (Ozes et al., 1999; Romashkova and Makarov, 1999; Foehr et al., 2000), although this is discussed controversially in the literature (Béraud et al., 1999; Madge and Pober, 2000; Rauch et al., 2000). As serine phosphorylation of IκBα and degradation of IκBs α and β were not detected in PC12 and SH-SY5Y cells in response to NGF, it is conceivable that activation of IκB kinases via Akt kinase or the Raf/ERK kinase pathway (Nakano et al., 1998; Nemoto et al., 1998) contributed little to NGF-induced NF-κB activation in our study. Interestingly, it has recently been demonstrated that the Raf/ERK pathway negatively regulates NFκB-dependent gene expression in cultured fibroblasts (Carter and Hunninghake, 2000), a potential mechanism for the decline in Bcl-xL expression in cultures treated with higher NGF concentrations.

In summary, our study demonstrates that NGF-induced NFκB activity and Bcl-xL expression require tyrosine phosphorylation of IκBα. Activation of NFκB via different ligand-receptor systems and downstream signal transduction pathways may enable the nervous system to maintain constitutive NFκB activity while responding with an increased NFκB activity during injury, inflammation, or repair processes. Moreover, the nonredundancy of NFκB activation pathways emphasizes the importance of this transcription factor for neuronal survival.

The authors thank Drs. M. Poppe, B. Levkau, and C.M. Luetjens for valuable advice and help with experiments, E. Bauerbach for expert technical assistance, Prof. C.B. Thompson for providing Bcl-x antiserum, Prof. D.W. Ballard for providing plasmid pIκBα, and Dr. H. Wajant for plasmid pTRAF6 dn.

This work was supported by the Deutsche Forschungsgemeinschaft (Pr 338/8-1).

Submitted: 8 September 2000
Revised: 4 January 2001
Accepted: 12 January 2001

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