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Role of IκBα and IκBβ in the Biphasic Nuclear Translocation of NF-κB in TNFα-Stimulated Astrocytes and in Neuroblastoma Cells

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ABSTRACT In infectious diseases of the central nervous system astrocytes respond to inflammatory cytokines like tumor necrosis factor α (TNFα) by activation of the transcription factor NF-κB, mediated by the proteolysis of its inhibitors IκBα and IκBβ. We studied the kinetics of NF-κB induction by TNFα in primary astrocytes, and in the neuroblastoma cell line Neuro2A, and compared it to fibroblasts. In the latter, NF-κB DNA binding activity was induced at 30 min and remained constant up to 4 h. In contrast, in astrocytes and in Neuro2A cells NF-κB DNA binding activity followed a biphasic pattern: it was induced after 30 min (early phase), declined after 1 h, and increased again at 2 to 4 h (late phase). The early phase was due to rapid degradation of IκBα. After 1 h IκBα was resynthesized to levels exceeding the amounts present in unstimulated cells. This paralleled the low levels of nuclear NF-κB binding activity. The decrease was not observed when IκBα resynthesis was inhibited by cycloheximide. Degradation of both IκBα and IκBβ contributed to the late phase of induction. However, the second peak occurred also in the absence of IκBβ proteolysis, demonstrating the importance of IκBα in the formation of the biphasic nuclear translocation of NF-κB. *GLIA 26:212–220, 1999.*

INTRODUCTION

Brain development and functions depend on the coordinated expression of different genes controlled by a variety of transcription factors, among them NF-κB (nuclear factor κB). In the brain, members of the NF-κB family of transcription factors have been identified in neurons, astrocytes, microglial cells, and glioblastoma cells (Korner et al., 1989; Sparacio et al., 1992; Bakalkin et al., 1993; Massa et al., 1993; Moynagh et al., 1993, Kaltschmidt et al., 1994; Perez-Otaño et al., 1996;). In neurons, NF-κB is either constitutively active (Kaltschmidt et al., 1994) or it is inducible (Guerrini et al., 1995; Kaltschmidt et al., 1995). In infectious diseases of the central nervous system, microglial cells and astrocytes interact with the immune system by producing cytokines and by responding to T-cell derived signals. Treatment of astrocytes with the inflammatory cytokines interleukin-1 and tumor necrosis factor α (TNFα) activates NF-κB (Moynagh et al., 1993; Sparacio et al., 1992).

In most cell types, NF-κB mediates an immediate early response by activating the transcription of numerous genes encoding cell adhesion molecules, cytokines, chemokines, and acute phase proteins. Stimuli activating NF-κB often represent pathogenic conditions such as viruses, bacteria, cytokines, oxidative stress, and UV irradiation (Baeuerle and Henkel, 1994; Grilli et al., 1993). NF-κB is composed of homo- and heterodimeric complexes of members of the Rel/NF-κB family of proteins, comprising p50, p65 (RelA), c-Rel, p52, and RelB. These proteins share a conserved region, the Rel

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homology domain, which contains sequences responsible for DNA binding, dimerization, and nuclear localization (Baldwin, 1996). The best characterized NF-κB complex is composed of a p50 and a p65 subunit. In most cells an inactive form of NF-κB is sequestered in the cytoplasm by an inhibitor, IκB. A variety of stimuli result in the phosphorylation by a recently identified kinase (reviewed in Baeuerle, 1998) and ubiquitination of the inhibitor. Its degradation by proteasomes allows NF-κB to translocate to the nucleus (reviewed in Baldwin, 1996). In the family of the IκBs, the most important appear to be IκBα and IκBβ (Beg and Baldwin, 1993; Verma et al., 1995). IκBα (37 kDa) is rapidly degraded by all known inducers, resulting in a fast accumulation of NF-κB in the nucleus. Nuclear NF-κB can activate the IκBα gene leading to the rapid reappearance of the protein in the cytoplasm where it again associates with NF-κB (Baeuerle and Henkel, 1994). Besides its function in the cytoplasm, IκBα can enter the nucleus where it binds to NF-κB. This complex is then transported back to the cytoplasm, reducing the nuclear NF-κB activity (Arenzana-Seisdedos et al., 1997). Continuous degradation of IκBβ (46 kDa) is thought to be responsible for a persistent activation of NF-κB (Thompson et al., 1995). Newly synthesized IκBβ accumulates then as an unphosphorylated protein in the cytoplasm, associates with NF-κB but does not mask its nuclear localization signal. Therefore, the NF-κB-IκBβ complex is translocated to the nucleus where it can bind to DNA (Suyang et al., 1996; Tran et al., 1997).

In view of the complexity of the Rel/NF-κB system, it is important to define the biological relevance of the inhibitors in different systems. We studied the roles of IκBα and IκBβ in NF-κB activation in a model system using TNFα-stimulated astrocytes, which are the primary cytokine-responsive cell in the brain. We observe a biphasic induction of NF-κB binding activity with a first increase at 30 min, a decrease at 1 h and a second increase at 2 to 4 h. Further, we find that IκBα is the key factor for the formation of this biphasic NF-κB activation.

MATERIALS AND METHODS

Cell Culture and Reagents

Primary astrocytes were prepared from newborn ICR mice as described (Frei et al., 1987) and were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gaithersburg, MD), supplemented with 5% heat-inactivated fetal calf serum (FCS). Neuro2A cells were obtained from ATCC (Rockville, MD) and were grown in minimum essential medium (MEM, Gibco-BRL, Gaithersburg, MD) with 10% FCS and 0.1mM nonessential amino acids. NIH3T3 fibroblast cells were grown in DMEM/10% FCS. All media were supplemented with 2 mM L-glutamine and antibiotics (penicillin/streptomycin). Recombinant mouse TNFα (Boehringer Mannheim, Mannheim, Germany) was added to a final concentration of 10 ng/ml and cells were incubated at 37°C for the indicated time periods. N-acetyl-Leu-Leu-norleucinal (ALLnL, 200 μM, Sigma Chemical Co., St. Louis, MO) and N-tosyl-L-lysine chloromethyl ketone (TLCK, 100 μM, Sigma Chemical Co., St. Louis, MO) were added 1 h before stimulation of cells with TNFα. Cycloheximide (25μg/ml) and H_2O_2 (50 μM) were from Fluka (Buchs, Switzerland). Astrocytes and Neuro2A cells were stimulated with H_2O_2 in minimum essential medium/10% FCS.

Nuclear and Cytoplasmic Extracts

Cells were placed on ice, washed twice with ice-cold phosphate-buffered saline (PBS), and collected by scraping. After pelleting, cells were resuspended in 5 packed cell volumes (pcv) of buffer A (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40 (NP-40), aprotinin (1 μg/ml), leupeptin (10 μg/ml), 5 mM sodium fluoride, and 1 mM β-glycerophosphate). Cells were lysed on ice for 2 min and complete lysis was verified with a microscope. Nuclei were spun down, and the supernatant was saved as cytosolic fraction after the addition of glycerol (25% final concentration). The nuclear pellet was resuspended in 2 pcv of buffer C [20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, aprotinin (1 μg/ml), leupeptin (10 μg/ml), 5 mM sodium fluoride, 1 mM β-glycerophosphate, and 25% glycerol], rotated for 30 min at 4°C, and spun for 20 min at 14,000 rpm in a microcentrifuge. The supernatant was used as nuclear extract. Protein concentrations in nuclear and cytoplasmic extracts were measured by the Bradford assay (Bio-Rad, Richmond, CA).

Electrophoretic Mobility Shift Assay (EMSA)

For NF-κB binding reactions nuclear extracts (10 μg) were incubated with 10,000 c.p.m. of a ^{32}P-labelled probe containing the κB site from the immunoglobulin κ light-chain gene (Zabel et al., 1991) in a buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 4% Ficoll, and 2 μg poly (dI-dC) in a final volume of 15 μl for 20 min at room temperature. The binding reactions detecting Oct-1 were performed in a buffer containing 30 mM KCl, 4 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, 4% Ficoll, 2 μg poly (dI-dC), and 10,000 c.p.m. of a ^{32}P-labelled probe containing the octamer binding site (Promega, Madison, WI). DNA-protein complexes were separated from unbound DNA on native 4% polyacrylamide gels in 0.5×TBE buffer at 10V/cm for 2 h at room temperature. Gels were vacuum dried, exposed on a PhosphorImager screen and DNA-protein complexes were quantified. Competition experiments were done by mixing a 10-fold or 50-fold molar excess of competi-

tor DNA to the binding reaction before adding the nuclear extract. The oligonucleotide competitors had the following sequences: wild-type AGTTGAGGGACTTTCCCAGG; mutant AGTTGAGGcGACTTTCCCAGG.

Antibody supershift experiments were performed by adding 2 µl of affinity-purified polyclonal antibodies to the reaction mixture, and incubating for 30 min on ice before addition of the radiolabeled probe. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) (anti-p65: sc-372, anti-p50: sc-114, anti-RelB: sc-226, anti-p52: sc-297, and anti-cRel: sc-70). Treatment of nuclear extracts with the detergent sodium deoxycholate (DOC) was performed as described (Beg et al., 1993). Briefly, DOC was added to a concentration of 0.8% and incubated for 5 min, after which NP-40 was added to a concentration of 1.2% and further incubated for 5 min. Radiolabeled probe and poly (dI-dC) were then added, and the binding reactions incubated for 15 min before being loaded onto gels.

Western Immunoblotting

Nuclear or cytoplasmic extracts (10 µg) were fractionated on 10% polyacrylamide-SDS gels and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). After blocking with 0.2% I-Block (Tropix), 1% goat serum in PBS, the membranes were incubated for 1 h at room temperature with affinity-purified rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for p65 (sc-372, 1:10,000), IκBα (sc-371, 1:1,000), or IκBβ (sc-945, 1:1,000). Immune complexes were detected by binding peroxidase-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO, 1:10,000) for 1 h at room temperature and analyzed with the enhanced chemiluminescence assay (ECL; Amersham, Arlington Heights, IL). After completion of the immunoblotting experiment, membranes were incubated with amido black to verify equal loading and transfer.

RESULTS

Biphasic Induction of NF-κB DNA Binding in the Nucleus of Astrocytes and Neuro2A Cells

In order to investigate the kinetics of NF-κB activation, primary astrocytes, the neuroblastoma cell line Neuro2A and NIH3T3 fibroblast cells were exposed to TNFα (10 ng/ml) for various times and nuclear extracts were analyzed in electrophoretic mobility shift assays (EMSA) with a radiolabeled oligonucleotide containing an NF-κB binding site. The activity of the nuclear extracts was controlled by using a DNA probe containing the binding site for the Oct-1 transcription factor (Fig.1A, second panel). In astrocytes and Neuro2A cells the NF-κB complex was induced in a biphasic pattern (Fig.1A, upper panel, lanes 1 to 10). The first peak occurred at 30 min and showed a 4-fold induction relative to extracts from unstimulated cells. After 1 h the amount of NF-κB binding activity decreased, increased again at 2 h, and persisted up to 4 h. The second peak reached about the same levels as the first peak. This biphasic induction was also observed in cultures of primary cerebellar neurons (data not shown). In contrast, in NIH3T3 cells the amount of NF-κB binding increased 11-fold after 30 min and remained approximately constant up to 4 h (lanes 11 to 15). The same monophasic pattern was also observed in other cells not derived from brain, such as HeLa, COS, and LM (data not shown).

Binding specificity of the NF-κB complex was demonstrated using a 10-fold or a 50-fold excess of a wild-type oligonucleotide which competed for binding (Fig. 1B, lanes 1 to 3); the same amount of a mutated oligonucleotide had no such effect (lanes 4 and 5). To analyze the composition of the NF-κB complex, gel mobility supershift assays were performed with antibodies against NF-κB subunits. Addition of anti-p65 or anti-p50 antibodies resulted in the loss or a strong reduction, respectively, of the NF-κB complex and the appearance of a supershifted band (Fig. 1C, lanes 2 and 3). Antibodies against RelB, p52 and cRel had no such effect (lanes 4 to 6).

We then examined if the biphasic and monophasic NF-κB DNA binding pattern is reflected by the amount of IκBα in the cytoplasm. Western immunoblotting experiments using cytoplasmic extracts of TNFα stimulated astrocytes and Neuro2A cells showed that IκBα was almost completely degraded after 30 min, coinciding with the early peak in NF-κB DNA binding. The inhibitor was resynthesized after 1 h and reached levels exceeding the amounts present in unstimulated cells (Fig.1A, bottom panel). The IκBα overproduction paralleled the low NF-κB binding activity observed at one hour. After 2 and 4 h induction IκBα levels were again reduced. In NIH3T3 cells IκBα was degraded after 30 min and the protein levels remained low for at least 4 h (lanes 11 to 15).

The strong overexpression of IκBα after 1 hour prompted us to investigate if NF-κB DNA binding was influenced when IκBα resynthesis was blocked by the protein synthesis inhibitor cycloheximide. In the presence of 25µg/ml cycloheximide the NF-κB DNA binding profile induced by TNFα did not show a decrease at 1 h in astrocytes (Fig. 1D); IκBα was degraded after 30 min and was not resynthesized later (bottom panel). Thus, interference with IκBα resynthesis prevents the reduction of NF-κB binding activity at 1 h and thereby the development of a biphasic pattern.

Nuclear Translocation of NF-κB Reflects Its Biphasic DNA Binding Pattern

Newly synthesized IκBα can enter the nucleus and associate with NF-κB. This process prevents NF-κB from binding to DNA (Arenzana-Seisdedos et al., 1995) or even removes NF-κB from postinitiation complexes

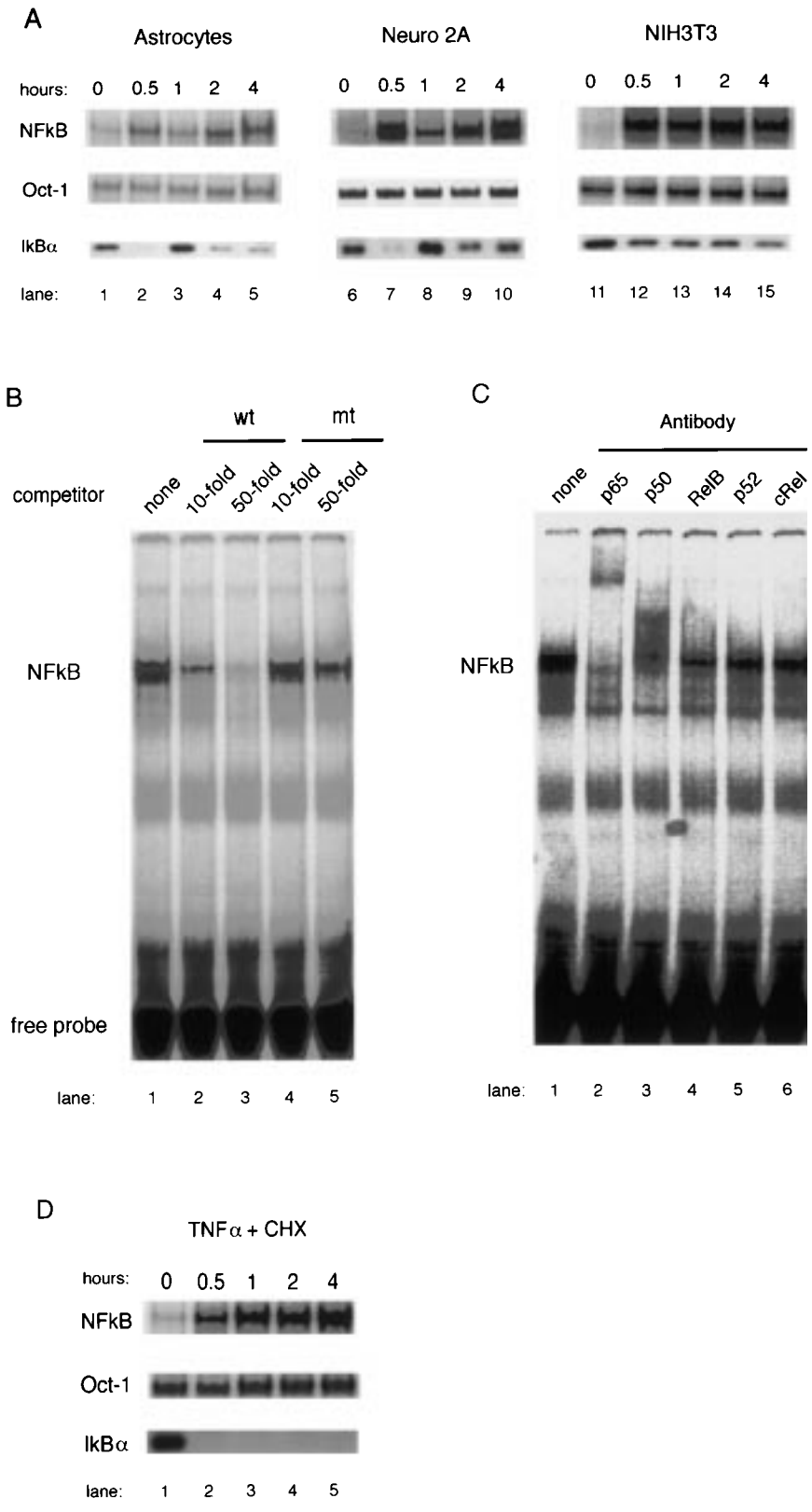

Fig. 1. **A:** TNFα induces a biphasic NF-κB DNA binding pattern in astrocytes and Neuro2A cells, but not in fibroblasts. Astrocytes, Neuro2A cells, and NIH3T3 fibroblasts were stimulated with TNFα (10 ng/ml) for the indicated times. Nuclear extracts (10 μg) were used for EMSA using either an NFκB binding site (upper panel) or an octamer binding site (middle panel) as a DNA probe. The NF-κB complex was detected at the following levels (relative to control levels): Astrocytes 4.1 (30 min), 2.1 (1 h), 3.6 (2 h), and 3.7 (4 h); Neuro2A cells 3.7 (30 min), 2 (1 h), 3.4 (2 h), and 3.5 (4 h); NIH3T3 11.5 (30 min), 9.6 (1 h), 11.1 (2 h), and 11.0 (4 h). Ten micrograms of cytoplasmic extracts were used for Western immunoblots with IκBα antibodies (lower panel). **B:** EMSA with 10 μg nuclear exctracts from astrocytes treated for 30 min with TNFα in the absence of competitor DNA (lane 1) or in the presence of a 10-fold or 50-fold molar excess of a wild-type competitor oligonucleotide (lanes 2 and 3) or of a mutant competitor oligonucleotide (lanes 4 and 5). **C:** The predominant NF-κB complex in astrocytes consists of a p65/p50 homodimer. Gel mobility supershift experiments with 10 μg nuclear extracts from astrocytes treated with TNFα for 30 min in the absence of antibody (lane 1) or after the addition of subunit-specific antibodies (lanes 2 to 6). **D:** EMSA with 10 μg nuclear extracts from astrocytes treated for various times with 10 ng/ml TNFα in the presence of 25 μg/ml cycloheximide (CHX). Results with an NF-κB DNA probe (upper panel) and an octamer DNA probe (second panel) are shown. Cytoplasmic extracts were used for Western immunoblots with IκBα antibodies (lower panel).

(Tran et al., 1997). Such a mechanism may be responsible for the termination of NF-κB DNA binding in the first post induction phase in astrocytes. If IκBα were able to associate with NF-κB in the nucleus, it should be possible to release NF-κB from its inhibitor by treatment of nuclear NF-κB-IκBα complexes with the detergent deoxycholate (DOC) and NP40 (Baeuerle and Baltimore, 1988).

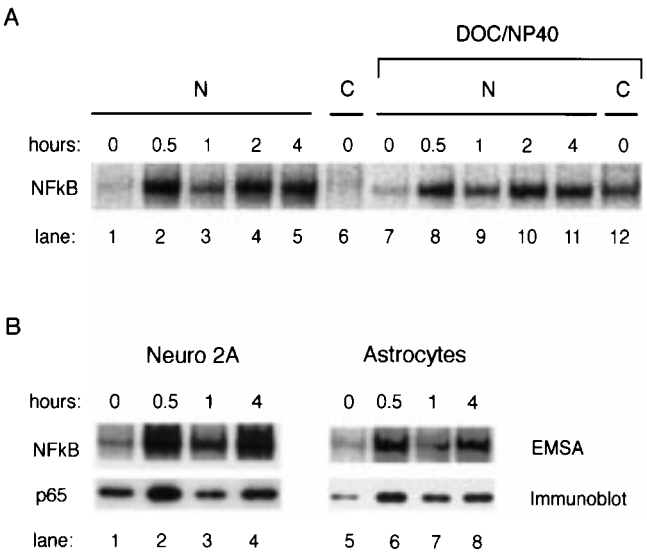

Fig. 2. **A:** NF-κB nuclear translocation coincides with the biphasic DNA binding pattern. Astrocytes were treated with 10 ng/ml TNFα for the indicated times. An EMSA was performed with nuclear (N, lanes 1 to 5 and 7 to 11) or cytoplasmic (C, lanes 6 and 12) extracts, which were either left untreated (lanes 1 to 6) or treated with 0.8% DOC/ 1.2%NP40 (lanes 7 to 12) before adding the radiolabeled probe. **B:** Nuclear extracts from TNFα stimulated Neuro2A cells (lanes 1 to 4) or astrocytes (lanes 5 to 8) were used for EMSA with an NF-κB binding site (upper panel) and for a Western immunoblot with p65 antibodies (lower panel).

To address this question, we treated nuclear extracts from TNFα induced astrocytes with 0.8% DOC/1.2% NP40 (Fig. 2A, lanes 7 to 11) and compared them to untreated extracts in EMSA (lanes 1 to 5). No increase in DNA binding activity could be seen in the detergent treated samples, most importantly not at 1 h, indicating that IκBα did not enter the nucleus and inhibit NF-κB DNA binding activity. As a control, cytoplasmic extracts from unstimulated cells were detergent treated and IκBα was released from the NF-κB complex resulting in an increase in DNA binding activity (compare lanes 6 and 12).

Another explanation for the low amount of NF-κB DNA binding activity after one hour TNFα stimulation would be that NF-κB protein is temporarily hindered to translocate to the nucleus. An immunoblotting experiment was performed to determine the total amount of p65 protein present in the nucleus of TNFα treated Neuro2A cells (Fig. 2B, lanes 1 to 4) or astrocytes (lanes 5 to 8). The lower panel shows a strong increase in nuclear p65 levels after 30 min and a marked decrease after 1 h. This parallels the NF-κB DNA binding activity observed in EMSA (upper panel) and confirms that the reduction of the NF-κB DNA binding activity in the early phase is due to an inhibition of nuclear translocation of NF-κB.

Role of IκBα and IκBβ Proteolysis in the Late Phase of NF-κB Translocation

Peptide aldehydes, a class of protease inhibitors interfering with proteasome functions, block the degradation of IκBα and the nuclear appearance of NF-κB (Baldwin, 1996). We reasoned that if such an inhibitor would interfere with either IκBα or IκBβ proteolysis we might determine their individual roles in the biphasic NF-κB activation. We thus treated astrocytes and NIH3T3 cells with either TNFα alone or with TNFα and the proteasome inhibitor N-acetyl-Leu-Leu-norleucinal (ALLnL, Fig. 3). Astrocytes treated only with TNFα showed again a biphasic induction of NF-κB DNA binding activity (Fig. 3A, upper panel, lanes 1 to 5); IκBα was rapidly degraded and resynthesized to high abundance (third panel from the top). IκBβ was detected as two electrophoretically different forms (lanes 4 and 5). Treatment of extracts with alkaline phosphatase resulted in the disappearance of the upper form and the enhancement of the lower form; this effect was

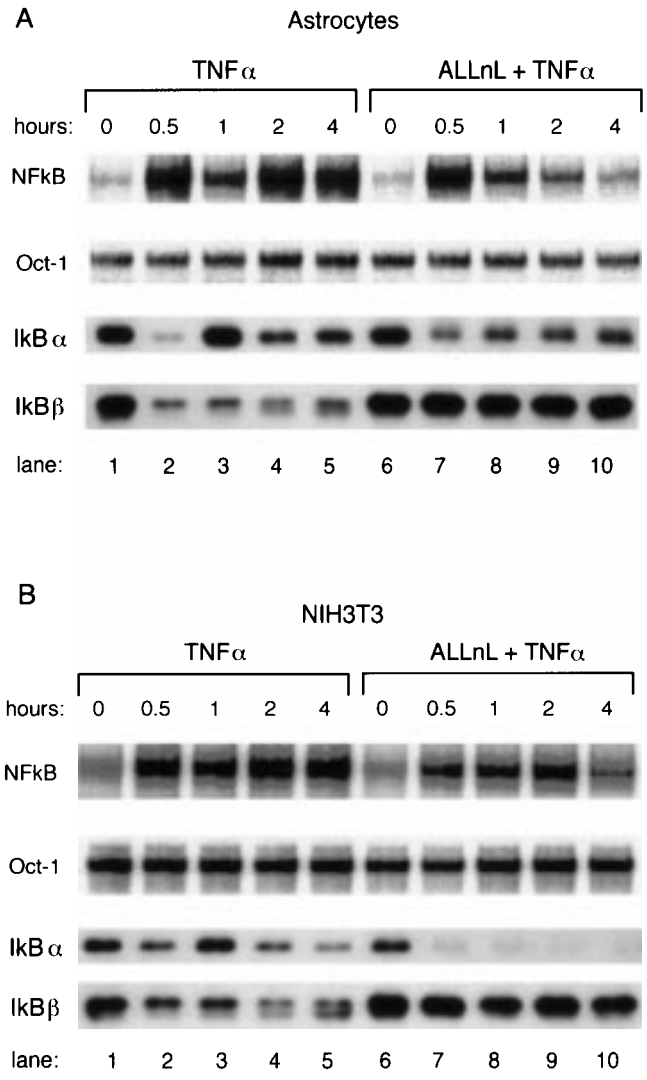

Fig. 3. The proteasome inhibitor ALLnL inhibits the late phase of NF-κB DNA binding. Astrocytes (**A**) or NIH3T3 cells (**B**) were either left untreated (lanes 1 to 5) or pretreated with 200 μM ALLnL for one hour (lanes 6 to 10) before stimulation with TNFα. EMSA was performed with nuclear extracts and a radiolabeled NF-κB probe (upper panel) or an octamer probe (second panel). Cytoplasmic extracts were used for Western immunoblots with IκBα antibodies (third panel) or IκBβ antibodies (lower panel).

blocked by phosphatase inhibitors (data not shown). Hence, IκBβ exists as a slower migrating phosphorylated form and a faster migrating unphosphorylated variant (Suyang et al., 1996). Upon TNFα induction the phosphorylated IκBβ protein was rapidly degraded and remained at constantly low levels, whereas the unphosphorylated variant was not detectable after 30 min stimulation but thereafter progressively accumulated to low levels (lowest panel). When astrocytes were pretreated for 1 h with 200 μM ALLnL and then exposed to TNFα, NF-κB DNA binding activity was monophasic with an increase at 30 min, a decrease after one hour, but no second peak at later time points (upper panel, lanes 6 to 10). ALLnL did not inhibit IκBα degradation, but appeared to interfere with IκBα resynthesis (second lowest panel). In contrast, IκBβ proteolysis was completely blocked by the proteasome inhibitor (lowest panel). This shows that the early phase of induction can also occur without IκBβ degradation.

When NIH3T3 cells were incubated with ALLnL and then stimulated with TNFα, the NF-κB DNA binding pattern remained monophasic (Fig. 3B, upper panel, lanes 6 to 10). It is noticeable that nuclear extracts from cells treated with the inhibitor and TNFα showed much lower DNA binding activity at 4 hours than extracts from cells treated only with TNFα (compare lanes 5 and 10). As true for astrocytes, signal induced proteolysis of IκBα was not affected in NIH3T3 cells by ALLnL. However, the protein was not resynthesized (second lowest panel). IκBβ degradation was completely inhibited (lowest panel).

In the light of the NF-κB binding pattern being biphasic in astrocytes and monophasic in NIH3T3 cells it is remarkable how similar the IκBα and IκBβ profiles are in astrocytes and NIH3T3 cells upon TNFα induction. The observation that in both cell types the inhibitor ALLnL drastically decreased the level of NF-κB DNA binding activity at 4 h of TNFα induction and blocked the degradation of IκBβ points to an important role of IκBβ in the induction of the second peak in astrocytes.

The serine protease inhibitor TLCK (N^a-tosyl-L-lysine chloromethyl ketone) prevents TNFα induced phosophorylation and degradation of IκBα in HeLa and Jurkat T cells and thereby inhibits the induction of NF-κB activity (Finco et al., 1994; Henkel et al., 1993; Miyamoto et al., 1994). It is not known if TLCK also interferes with IκBβ proteolysis. To further assess the involvement of IκBβ degradation in the biphasic induction profile of NF-κB, astrocytes were pretreated with 100 μM TLCK for 1 h before adding TNFα. The data showed an induction of NF-κB DNA binding activity at 30 min, followed by constantly lower levels at 1 to 4 h (Fig. 4A, upper panel). IκBα proteolysis and resynthesis was not affected in astrocytes at an inhibitor concentration active in HeLa or Jurkat T cells (third panel). However, TLCK itself resulted in the disappearance of part of the phosphorylated slower migrating variant of IκBβ (lowest panel, lanes 1 and 2) and the appearance of the unphosphorylated faster migrating IκBβ form,

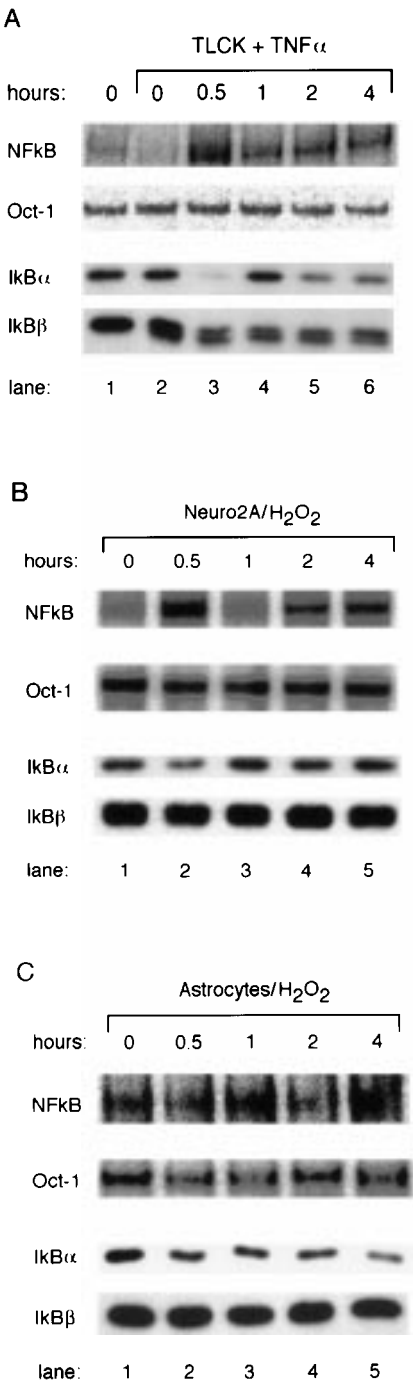

Fig. 4. **A:** The protease inhibitor TLCK inhibits the late phase of NF-κB DNA binding. Astrocytes were either left untreated (lane 1) or pretreated with 100 μM TLCK for 1 h (lanes 2 to 6) before stimulating with TNFα (lanes 3 to 6). **B,C:** Hydrogen peroxide induces a biphasic NF-κB nuclear translocation in the absence of IκBβ degradation. Neuro2A cells (B) or astrocytes (C) were treated with 50 μM or 200 μM H_2O_2, respectively, for various times and nuclear and cytoplasmic extracts were prepared. In A,B, and C, EMSA was performed with nuclear extracts and a radiolabeled NF-κB probe (upper panel) or an octamer probe (second panel). The NF-κB complex was detected at the following levels (relative to control levels): (B) Neuro2A 4.64 (30 min), 1.22 (1 h), 2.14 (2 h), 2.5 (4 h); (C) Astrocytes 1.38 (30 min), 2.14 (1 h), 0.95 (2 h), 1.75 (4 h). Cytoplasmic extracts were used for Western immunoblots with IκBα antibodies (third panel) or IκBβ antibodies (lower panel).

leading to an approximately equal ratio of the two variants (lane 2). Upon TNFα treatment both forms were degraded and their amounts remained constant up to 4 h (lanes 3 to 6). In astrocytes treated only with TNFα, equivalent ratios of the two IκBβ forms were reached only after 4 h (compare Fig. 4A, lane 2 with Fig. 3A, lane 5). This experiment shows that NF-κB released from IκBβ during the TLCK pretreatment period (lanes 1 and 2) or during the first 30 min of TNFα induction (lanes 2 and 3) cannot be relevant for the late phase of NF-κB translocation.

In neurodegenerative or infectious diseases of the central nervous system, both neurons and glial cells produce cytokines, neurotrophins and reactive oxygen species, such as hydrogen peroxide (H_2O_2; Fontana et al., 1996). Treatment of some cell lines, e.g., Jurkat T cells and HeLa cells (Schreck et al., 1992) with H_2O_2 can activate NF-κB by producing oxygen radicals in the cell. We therefore determined the NF-κB DNA binding profile of Neuro2A cells and astrocytes exposed to H_2O_2. As true for TNFα, H_2O_2 (50 μM) induced a biphasic activation pattern of NF-κB in Neuro2A cells (Fig. 4B, upper panel). As expected, IκBα was degraded and resynthesized (third panel). However, unlike the effect of TNFα, H_2O_2 did not lead to proteolysis of IκBβ (lowest panel). Similarly, exposure of astrocytes to H_2O_2 (200 μM) resulted in a biphasic activation of NF-κB (Fig. 4C). The first peak occurred later than in Neuro2A cells, namely after 1 h. The binding activity decreased at 2 h and increased again at 4 h. IκBα was degraded, whereas IκBβ was not, as seen with Neuro2A cells. These findings indicate that the second peak of the NF-κB DNA binding activity must be due to NF-κB protein released from IκBα and not from IκBβ. Furthermore, these data confirm the role of IκBα in terminating NF-κB activation at 1 h, resulting in a biphasic induction pattern. It cannot be excluded that IκBβ contributes to the late phase of induction, since the levels of NF-κB binding activity induced by H_2O_2 at 2 and 4 h are much lower than in the experiment where NF-κB was induced by TNFα (Fig. 1).

To investigate if NF-κB regulated genes show a biphasic transcription pattern upon induction with TNFα, Northern blotting experiments were performed with selected probes. None of the genes tested (MIP-2, G-CSF, TNFα, IκBα, VCAM, and MHC class I) showed a biphasic transcription profile in TNFα-stimulated astrocytes (data not shown).

DISCUSSION

We show here that TNFα stimulation of astrocytes and of the neuroblastoma cell line Neuro2A leads to a biphasic induction of NF-κB DNA binding activity. An early peak occurs after 30 min; at 1 h DNA binding activity returns to basal levels, increasing again after 2 h. Gel mobility supershift experiments indicate that the NF-κB complex in astrocytes is composed of a p65/p50 heterodimer (Fig. 1C). This is in apparent contrast to results reported by Diehl et al. (1995), who interpret the lack of a supershift with an anti-p50 antibody as an indication that only a p65 homodimer exists in rat astrocytes. It is of note, that no positive control for p50 supershifts was shown by Diehl et al. (1995).

The biphasic nuclear translocation of NF-κB may be due to changes in IκBα and IκBβ levels, the most important inhibitors of p65/p50 heterodimers. It cannot be excluded that other IκB molecules might also play a role in the biphasic induction of NF-κB. However, we consider this unlikely, since IκBγ is limited to mouse B cells, IκBδ inhibits RelB/p52 complexes (Baldwin, 1996), and IκBε associates almost exclusively with RelA and cRel (Whiteside et al., 1997).

Recently, a biphasic activation of NF-κB was described in hepatocytes stimulated with TNFα (Han and Brasier, 1997). In agreement with our data on astrocytes, the proteasome inhibitor ALLnL interfered with IκBβ proteolysis and inhibited the late phase of NF-κB translocation. This was interpreted to mean that in hepatocytes the second peak may be due to IκBβ proteolysis (Han and Brasier, 1997). In astrocytes we believe that IκBα may be important for the biphasic induction since 1) H_2O_2 induces a biphasic nuclear translocation of NF-κB in the absence of IκBβ proteolysis (Fig. 4), 2) synthesis of IκBα is required for the decrease of NF-κB binding activity after 1 h (Fig. 1D), and 3) in the presence of the protease inhibitor TLCK the late phase of induction is abolished, although IκBβ is degraded to some extent (Fig. 4A). The second peak of induction observed in the hepatocyte system might also be explained by the dominant role of IκBα, rather than by IκBβ proteolysis (Han and Brasier, 1997).

We have seen that the termination of the first phase of induction is due to IκBα synthesis to levels exceeding those of unstimulated cells (Fig. 1D). This overproduction is probably due to increased transcription of the IκBα gene, which is activated by NF-κB (Baeuerle and Henkel, 1994) and whose promoter contains several NF-κB binding sites (Le Bail et al., 1993; Cheng et al., 1994; Ito et al., 1994). A role of IκBα in the control of the postinduction repression of NF-κB has also been demonstrated in fibroblasts from mice with a homozygous disruption of the IκBα gene, where NF-κB activity in the nucleus is prolonged upon TNFα stimulation (Beg et al., 1995; Klement et al., 1996). In astrocytes from wild-type mice a transient postinduction repression exists: NF-κB DNA binding levels increase again in the late phase of induction. Thus, the feedback system described above (Cheng et al., 1994; Ito et al., 1994; Le Bail et al., 1993) may be effective also in astrocytes.

In astrocytes and in hepatocytes (Han and Brasier, 1997) IκBα and IκBβ may be degraded by different pathways since the proteasome inhibitor ALLnL selectively blocks TNFα induced IκBβ degradation. ALLnL interference with IκBα resynthesis must be due to an indirect effect. One possible pathway for this effect may

be transcriptional regulation: the promoter of the IκBα gene contains putative binding sites for transcription factors other than NF-κB, e.g., SP1, interferon γ response element, AP1, AP2, and AP3 (Chiao et al., 1994). It is conceivable that one or more of these factors are targets of the proteasome. Inhibition of the proteasome complex would inhibit IκBα promoter activation and lead to reduced transcription.

The biphasic response of NF-κB has precedents among transcription factors. It has also been reported for AP-1 in endothelial cells exposed to shear stress (Lan et al., 1994), in the rat cerebral cortex after kainate administration (Unlap and Jope, 1994), and in HeLa cells which were exposed to hypoxia (Rupec and Baeuerle, 1995). AP-1 is induced at the transcriptional level and therefore the early and the late phase of the activation are dependent on protein synthesis (Rupec and Baeuerle, 1995). This is in contrast to the induction of NF-κB in astrocytes, where only the late phase depends on protein synthesis as shown by using cycloheximide as an inhibitor of IκBα resynthesis (Fig. 1D).

The significance of the biphasic activation of NF-κB is not known yet. In first experiments the TNFα induced expression of the MIP-2, G-CSF, TNFα, IκBα, VCAM, and MHC class I genes did not show a biphasic transcription pattern (data not shown). But similar kinetics of induction, repression and reinduction exist in two cell-types of the nervous system (astrocytes, Neuro2A cells). This may suggest that biphasic induction is crucial for the activation of genes which require a precise expression timing and do not tolerate prolonged deviations from homeostatic expression levels.

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