Molecular Medicine 5: 372-381, 1999

Molecular Medicine

Loss of NF-κB Activity during Cerebral Ischemia and TNF Cytotoxicity

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Abstract

Recent evidence implicates tumor necrosis factor (TNF), a cytokine with both cytotoxic and cytoprotective activities, in the pathogenesis of cerebral ischemia. The development of TNF cytotoxicity is dependent upon the balance between the activities of intracellular signaling pathways that mediate either apoptotic or anti-apoptotic effects. One critical protective signaling mechanism is the activation of nuclear factor (NF)-κB, a ubiquitous transcription factor that regulates expression of anti-apoptotic gene products. Here we show the distribution and kinetics of NF-κB activation and the correlation between loss of NF-κB activity, TNF up-regulation, and apoptosis in a standardized rat model of focal cerebral ischemia. We observed a rapid and progressive ischemia-induced loss of p65 immunoreactivity within the ischemic core and nearby penumbra. These findings were confirmed by Western blot analysis of nuclear extracts and by electrophoretic mobility shift assay. The anatomical area of suppressed NF-κB activity overlapped significantly with the zones of TNF overexpression and apoptosis. Loss of NF-κB activity and increased TNF expression preceded the onset of cell death. Direct evidence that loss of NF-κB activity can sensitize brain cells to TNF cytotoxicity was obtained in vitro by co-administration of MG-132, an inhibitor of NF-κB activation, and TNF to neuronal-like and glial-like cell cultures. Inhibition of NF-κB significantly increased the sensitivity of these cultures to TNF cytotoxicity, indicating that the observed loss of neuronal NF-κB activity during cerebral ischemia can participate in the development of TNF-induced cytotoxicity.

Introduction

The observation that cerebral ischemia induces increased expression of tumor necrosis factor (TNF), hydrogen peroxide, and glutamate, which induce apoptosis and activate nuclear factor (NF)-κB (1–7), suggests that NF-κB activation might mediate the development of neurodegeneration. Paradoxically, however, NF-κB has also been implicated in the induction of both anti-apoptotic (8–19) and pro-apoptotic states (1,20–24). It is unclear whether NF-κB activation participates in the development of cytotoxicity or functions as part of the protective signaling pathways that confer cellular defense.

NF-κB is a ubiquitous transcription factor comprising at least five DNA binding protein subunits. Classic NF-κB consists of two subunits, p50 and p65, which can bind to DNA as a homo- or heterodimer (25). At least two different cellular forms of NF-κB exist: an inactive, non-DNA-binding cytoplasmic form that is bound by inhib-

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itor proteins IκBα and IκBβ (reviewed in ref. 7), and an activated form that appears when IκB is released. Activated NF-κB translocates to the nucleus where it interacts with DNA-binding sites to regulate gene expression. NF-κB is activated by cytokines [TNF, interleukin-1β (IL-1β), IL-2, (LIF)], growth factors [nerve growth factor (NGF)], bacterial products [lipopolysaccharide (LPS), exotoxin B, TSST-1)], oxidants (hydrogen peroxide, ozone), physical stress (UV radiation, γ radiation) and viral infection (reviewed in ref. 26). After activation, NF-κB induces the expression of genes encoding cytokines, inflammatory enzymes, cell adhesion molecules, cell surface receptors, and acute-phase proteins. NF-κB is widely expressed in the central nervous system (CNS) as both a constitutively active and inducible gene regulator (4,20,27–29).

Recent evidence implicates NF-κB in signaling the cellular responses to TNF, a pleiotropic cytokine produced in response to infection, injury, or ischemia. Cerebral ischemia stimulates the induction of TNF mRNA and protein within 2 hr, prior to the development of significant cell death (30–34). Administration of TNF synthesis inhibitors (e.g., CNI-1493) or neutralizing anti-TNF antibodies confers significant protection from cerebral infarction (35–39). These observations suggest that TNF occupies a proximal position in the ischemic mediator cascade that causes neurodegeneration. Other evidence indicates that TNF cytotoxicity is significantly dependent upon a loss of NF-κB activity (40,41). For instance, treatment of NF-κB-deficient murine fibroblasts with TNF results in significantly increased cytotoxicity as compared to wild-type controls, and reintroduction of NF-κB confers protection against TNF cytotoxicity (9,11–15,42).

Although it can be difficult to dissect the overlapping and redundant mechanisms that characterize the pathobiological effects of a pleiotropic cytokine in vivo, we considered it plausible that underexpression of activated NF-κB in the ischemic cerebral cortex would influence neuron sensitivity to TNF-induced apoptosis. Accordingly, we examined the distribution and kinetics of NF-κB activity in the rat cerebral cortex after permanent middle cerebral artery occlusion (MCAO) using immunohistochemistry, Western blot analysis of nuclear extracts, and electrophoretic mobility shift assay. Cerebral ischemia induces a progressive loss of NF-κB activity in the ischemic cortex in a region that overlaps with both regions of maximal apoptosis and TNF overexpression. The loss of NF-κB and the increase in TNF expression precedes the development of significant cell death, and inhibiting NF-κB activation in neurons or glia in vitro significantly potentiates the cytotoxicity of TNF. Thus, ischemia-induced decreases in NF-κB activity can sensitize cells to the injurious effects of TNF.

Materials and Methods

Focal Cerebral Ischemia

Permanent focal cerebral ischemia was performed on male Lewis rats as described elsewhere (43). Briefly, the contralateral common carotid artery was occluded for 1 hr and the ipsilateral artery permanently occluded under ketamine anesthesia. The middle cerebral artery (MCA) was permanently occluded by electrocoagulation. Temperature was maintained about 37°C.

Tissue Preparation and Immunohistochemistry

At different time points after the onset of MCAO (2, 6, 24 hr, 3–15 days) animals were deeply anesthetized with an overdose of ketamine, and brains were removed and immediately frozen on dry ice. Ten-micrometer-thick sections were cut on a cryostat, air dried for 1 hr, fixed with absolute methanol for 10 min at −20°C, and again air dried. An activity-specific monoclonal anti-NF-κB antibody (Boehringer Mannheim) that recognizes the IκB-binding region on the DNA-binding p65 subunit (and thus selectively reacts with p65 in activated NF-κB) was employed for immunocytochemical characterization of NF-κB activation. After washing in phosphate-buffered saline (PBS) and incubating in a blocking solution [1% bovine serum albumin (BSA), and 0.3% Triton X-100 in 0.01 M PBS] for 1 hr at room temperature, sections were incubated for 2 to 24 hr at 4°C in a solution (5–10 μg/ml) of the activity-specific monoclonal anti-NF-κB antibodies in 0.3% Triton X-100/0.1% BSA/0.01 M PBS. Washed sections were then incubated with a solution (20 μg/ml) of bridging antibody (Boehringer Mannheim) for 1–2 hr at room temperature, washed again in PBS, and incubated for 30 to 45 min at room temperature with a 1:50 solution of APAAP (Boehringer Mannheim). After washing in PBS, sections were incubated in a freshly prepared and filtered substrate solution [1 Fast Red tablet (Boehringer Mannheim) in 2 ml of 100 mM Tris buffer, pH 8.2] until a clear visible color was developed. Sections were rinsed

with PBS and dH_2O and mounted with Crystalon.

TUNEL Staining

We used a terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) method for specific staining of DNA fragmentation in apoptotic cells as recommended by the manufacturer (ApopTag kit, Oncor). TUNEL-stained brain sections or cells were also stained with hematoxylin and eosin.

Preparation of Nuclear Extracts and Western Blot Analysis

Nuclear extracts were prepared as previously described (44) with modifications. Tissue samples from different parts of ischemic and nonischemic cortex from rats subjected to MCAO were frozen immediately in liquid nitrogen and then homogenized in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA in the presence of 1 mM DTT, 0.5 mM PMSF, 0.5 mg/ml benzamidine, 2 μg/ml leupeptin and aprotinin) and placed on ice for 10 min. Then extracts were treated with 1% Nonidet P-40. The nuclei were separated from cytosol by centrifugation at 20,000 × *g* for 15 min. At this point, supernatant (cytoplasmic extract) was removed and reserved at −80°C. Pellets were resuspended in buffer C (25% glycerol, 20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA 1 mM DTT, 0.5 mM PMSF, 0.5 mg/ml benzamidine, 2 μg/ml leupeptin and aprotinin) and incubated at 40°C for 30 min with stirring. This suspension was centrifuged 12,000 × *g* for 10 min. The supernatant was frozen as aliquots at −80°C. Protein concentration was determined by BCA Protein Assay Kit (Pierce). Immunoblot analysis was performed using enhanced chemiluminescence (Amersham Life Science). The primary rabbit polyclonal NF-κB p65 antibody (Santa Cruz, sc 362) was used at a dilution of 1:2000. The secondary antibodies were goat-anti-rabbit IgG conjugated to horseradish peroxidase (ICN/Cappel) at 1:1.000 dilution.

Electrophoretic Mobility Shift Assay (EMSA)

Double-stranded NF-κB oligonucleotides (Promega #E3291) were end labeled with T4 Polynucleotide Kinase (Promega #E3050) and [γ-^{32}P] ATP. For binding reactions, nuclear extracts (25 μg of protein) were incubated in buffer containing 5% glycerol, 20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 1 mM DTT, 1% NP-40, 2 μg of poly(dI-dC), and radiolabeled oligonucleotide (4.5×10^5 cpm) for 30 min in ice. Protein–DNA complexes were resolved on 7% native acrylamide gels run in 5 mM Tris (pH 8.3), 38 mM glycine until the dye front reached the bottom of the gel. The dried gels were exposed on a Molecular-Dynamics Phosphoimager Screen overnight.

Cell Culture

HTB11 and HTB14 cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% human serum, 10% penicillin-streptomycin (Sigma) and 100 mM sodium pyruvate (Sigma). Cells were treated with TNF at different concentrations (0.01–100 ng/ml for 24 hr) with and without nonspecific NF-κB inhibitor, MG-132 (10–20 μM; Calbiochem). The viability of cells was quantified by trypan blue assay (Sigma). TNF-induced apoptosis was detected by TUNEL staining.

Results

Cerebral Ischemia Suppresses NF-κB Activity

Using activity-specific anti-NF-κB antibodies that recognize only activated NF-κB by binding the IκB-binding region on the DNA-binding p65 subunit (Boehringer Mannheim), we observed constitutively expressed basal levels of activated NF-κB within neuronal nuclei throughout the brain cortex of normal and sham-operated rats. NF-κB immunoreactivity (IR) was highest within the cingulum, frontal and piriform cortex, and amygdala. These findings were specific, as nuclear NF-κB IR was not observed in the absence of primary antibodies. Within 2 hr after the onset of permanent MCAO, we observed a significant loss of nuclear NF-κB expression in the ischemic core and penumbra (Fig. 1). This loss of NF-κB is not attributed to cell death, as absence of nuclear NF-κB preceded the development of eosin-positive staining and TUNEL-positivity. The loss of NF-κB persisted for at least 24 hr, during the period in which the zone of neurodegeneration spread to involve a progressively larger area.

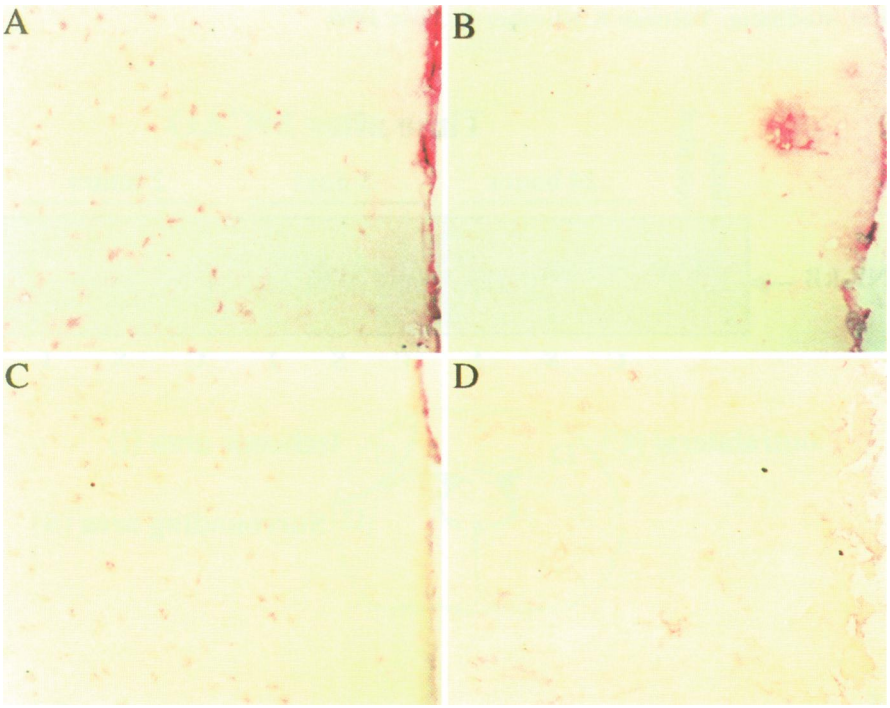


Fig. 1. Inhibition of NF-κB activity in the ischemic cortex after permanent focal cerebral ischemia. Brain sections were obtained at either 2 hr (A,B) or 24 hr (C,D) after the onset of MCAO. Note the loss of NF-κB activity within neuronal nuclei in the ischemic area (B,D) compared to the normally perfused contralateral side with constitutive NF-κB activity (A,C); also note the absence of NF-κB IR in cortical white matter.

Up-regulation of NF-κB Activity at 5 Days After the Onset of Ischemia

On the fifth day after MCAO, NF-κB IR in the region surrounding the infarction was more dense than in the normally perfused, contralateral cortex. Normal cortical white matter was devoid of activated NF-κB, as was the ischemic cortical white matter, for up to 5 days after the

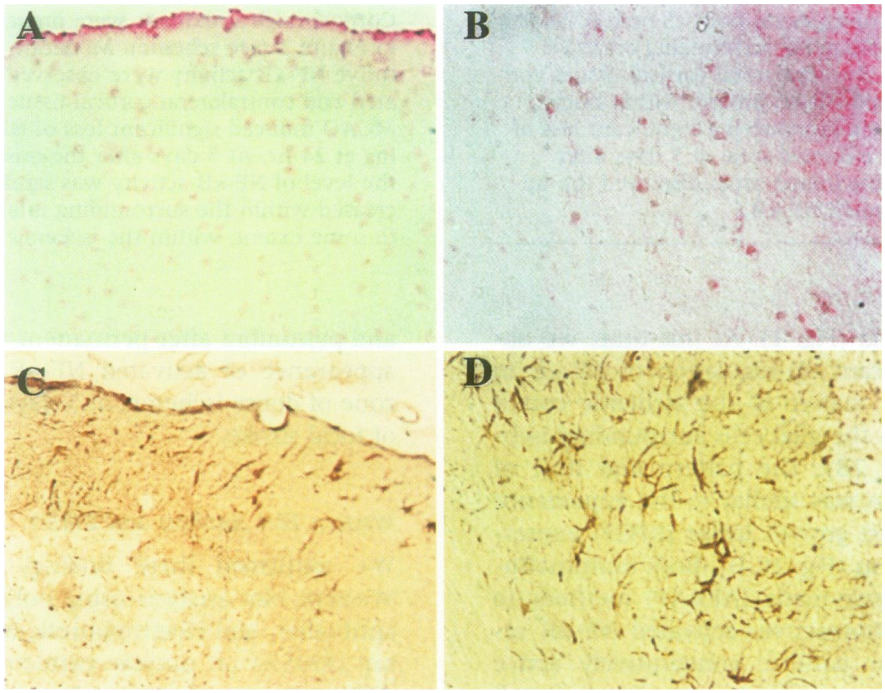
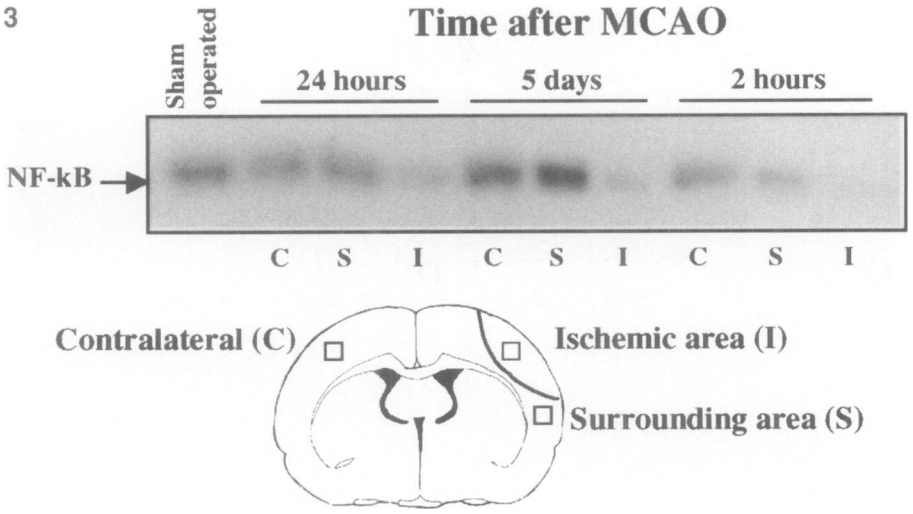


Fig. 2. Upregulation of NF-κB activity and bFGF expression at 5 days after MCAO. Note the appearance of NF-κB IR in the ipsilateral ischemic cortical white matter (A) and glial cells surrounding the ischemic core (right upper corner, B,D). bFGF IR (C,D) is correspondingly increased.



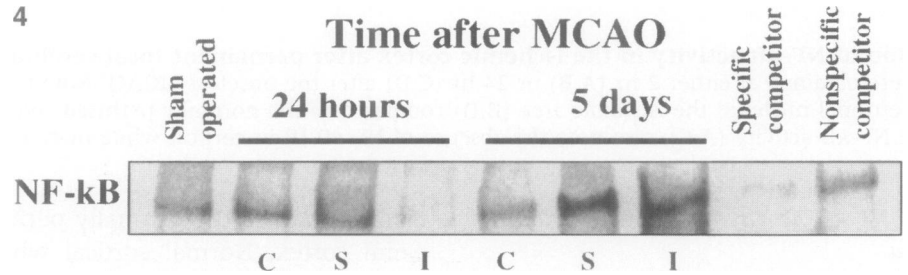


Fig. 3. Western blot analysis of nuclear extracts confirms loss of NF-κB activity, followed by later up-regulation in surrounding tissues. Cortical nuclear extracts prepared from ischemic (I) and contralateral nonischemic (C) zones, and from ipsilateral surrounding area (S). Sham-operated and contralateral cortical tissues constitutively expressed NF-κB protein within nuclei. Focal cerebral ischemia induced a significant loss of NF-κB within the ischemic area; at 5 days after MCAO, surrounding infarct areas displayed the up-regulation of activated NF-κB.

Fig. 4. Electrophoretic mobility shift assay (EMSA) of nuclear extracts confirms loss of NF-κB activity in ischemic cortex, followed by up-regulation at 5 days after onset of ischemia. Cortical nuclear extracts were prepared as described in Figure 3 (see scheme). Moderate levels of constitutive NF-κB activity were observed in sham-operated and contralateral cortical tissues (C). Permanent MCAO induced significant loss of NF-κB DNA-binding at 24 hr. At 5 days after the onset of ischemia, the level of NF-κB activity was significantly increased within the surrounding infarct areas (S) and, to some extent, within the ischemic zone (I).

onset of ischemia (Fig. 1). At this time, we observed a significant up-regulation of NF-κB in both the white matter of the ischemic cortex (Fig. 2A) and in the glial cells surrounding ischemic core (Fig. 2B). The pattern and time of NF-κB activation was similar to the appearance of basic fibroblast growth factor (bFGF) expression in these areas (Fig. 2C,D). Both immunofluorescence and enzymatic (APAAP) methods to visualize immunoreactive, activated NF-κB revealed a similar pattern: constitutively active NF-κB was present within normally perfused, nonischemic neuronal nuclei, but absent in white matter. This contrasted with the loss of neuronal NF-κB activity within the ischemic core and penumbra after permanent MCAO, and the appearance of activated NF-κB IR within the zone of dense ischemia at 5 days after induction of focal stroke.

Western Blot Analysis and EMSA

We confirmed these findings by performing Western blot analysis using polyclonal anti-p65 antibodies, and electrophoretic mobility shift assay (EMSA) on nuclear extracts prepared from different areas of cortex. Microdissected tissues were harvested from three sites: (*1*) the ischemic core and penumbra; (*2*) remote, normally perfused areas ipsilateral to MCAO; and (*3*) equiva-

lent anatomical areas from the contralateral side, and from matching anatomical areas of sham-operated animals. In agreement with the immunocytochemical data, Western blot analysis confirmed the constitutive expression of activated NF-κB in normally perfused, nonischemic cortical tissues (see Fig. 3). As early as 2 hr after the onset of ischemia, however, levels of activated NF-κB decreased significantly within the ischemic core as compared to within either the contralateral brain or to adjacent tissues. Decreased levels of NF-κB in the ischemic cortex were also observed at 24 hr and 5 days. Within 5 days after MCAO, however, NF-κB expression in tissues surrounding the ischemic area was increased compared to that in either the ischemic core or contralateral tissue. Similar patterns of NF-κB expression were observed using EMSA to detect DNA-binding protein specific for the NF-κB consensus sequence motif. Nuclear extracts prepared from cortical tissues after MCAO exhibited significantly decreased activity of NF-κB DNA-binding within the ischemic core and penumbra at 24 hr compared to that in sham-operated animals (see Fig. 4). Also in agreement with the immunohistochemistry and Western blot analysis of nuclear extracts, by day 5 we observed a significant increase in NF-κB-binding activity adjacent to the zone of dense ischemia, although NF-κB-binding activity within the ischemic core remained low.

Overlap of NF-κB Loss with Profound Apoptosis and TNF Upregulation

Previously we and others have reported that MCAO induces the appearance of increased TNF expression, and that the highest levels of TNF IR are found in the brain regions with the most extensive apoptosis (33). In the present study we found that the area of depressed NF-κB activity overlaps both the regions of up-regulated TNF expression as defined with monoclonal anti-TNF antibodies and the region of significant apoptotic cell death (Fig. 5). Thus, MCAO induces a rapid loss of activated NF-κB and a rapid increase in TNF expression in the ischemic core and penumbra that precedes the onset of apoptotic cell death in the same anatomic region.

Pharmacological Inhibition of NF-κB Activity Enhances Toxicity of TNF

To test the hypothesis that inhibition of NF-κB activity participates in TNF-mediated neurotoxicity, we measured the viability of human neuronal-like (HTB11) and glial-like (HTB14) cell cultures treated with TNF in the presence

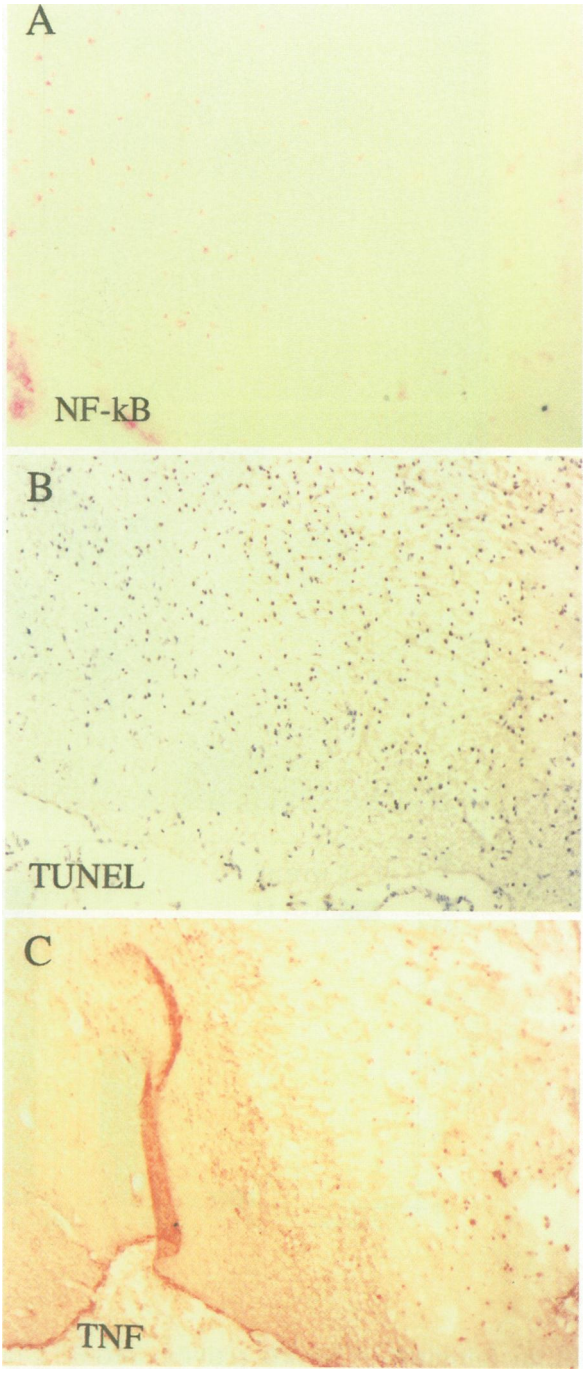


Fig. 5. Overlap between anatomic areas of decreased NF-κB activity, apoptotic cell death, and TNF overexpression. Note that 24 hr after MCAO, the loss of NF-κB activity within the ischemic area (A, activity-specific anti-p65 antibodies) overlapped with profound apoptotic cell death (B, TUNEL) and significant up-regulation of TNF expression (C, monoclonal anti-TNF antibodies). The ischemic area occupies the right side of each figure (A–C).

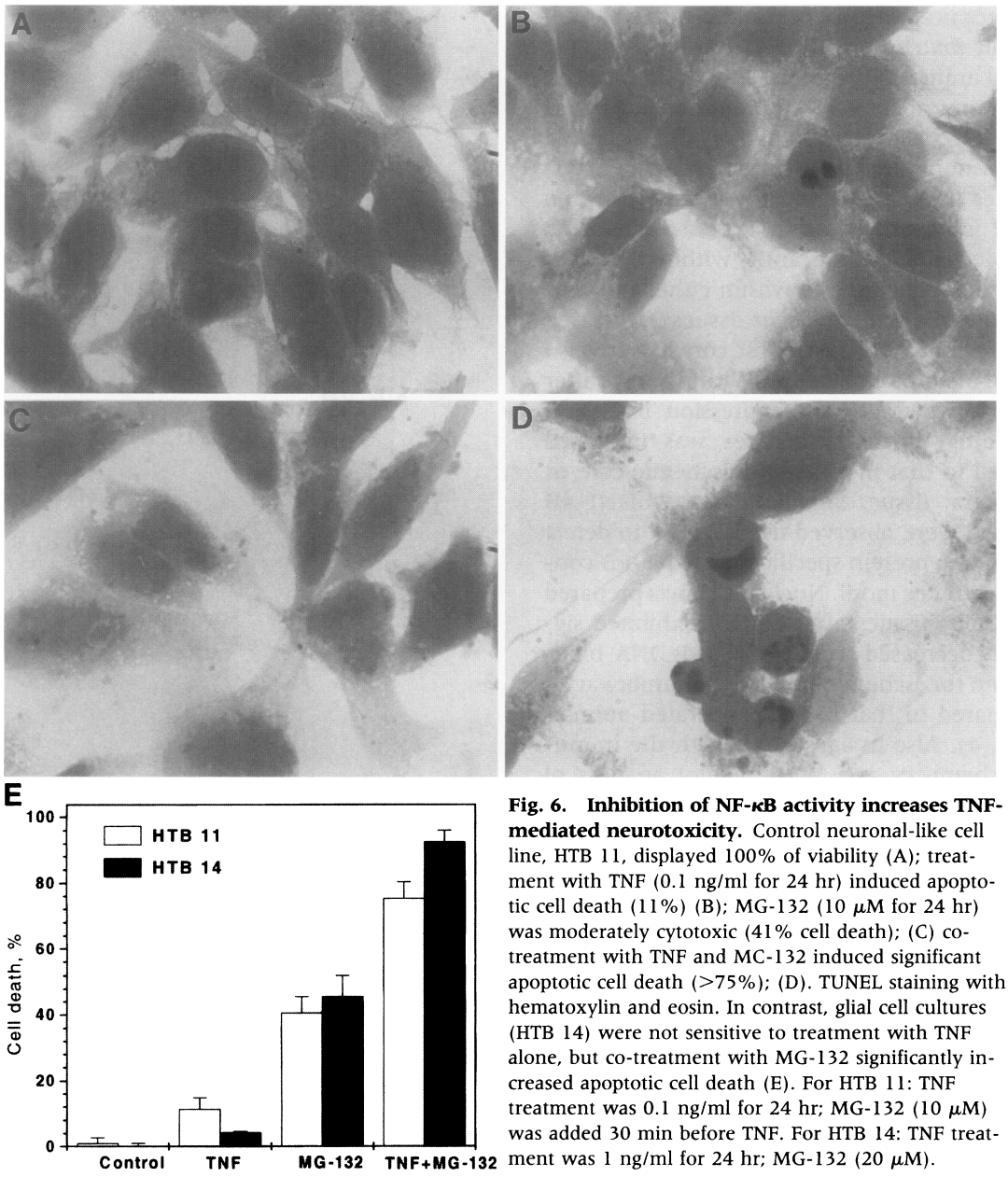


Fig. 6. Inhibition of NF-κB activity increases TNF-mediated neurotoxicity. Control neuronal-like cell line, HTB 11, displayed 100% of viability (A); treatment with TNF (0.1 ng/ml for 24 hr) induced apoptotic cell death (11%) (B); MG-132 (10 μM for 24 hr) was moderately cytotoxic (41% cell death); (C) co-treatment with TNF and MC-132 induced significant apoptotic cell death (>75%); (D). TUNEL staining with hematoxylin and eosin. In contrast, glial cell cultures (HTB 14) were not sensitive to treatment with TNF alone, but co-treatment with MG-132 significantly increased apoptotic cell death (E). For HTB 11: TNF treatment was 0.1 ng/ml for 24 hr; MG-132 (10 μM) was added 30 min before TNF. For HTB 14: TNF treatment was 1 ng/ml for 24 hr; MG-132 (20 μM).

of an inhibitor of NF-κB activity, MG-132 (a proteasome inhibitor, which prevents I-κB degradation). Addition of TNF (0.1 ng/ml, for 24 hr) to HTB11 cell cultures induced minimal dose-dependent cytotoxicity (11.3 ± 3.5% cell death; Fig. 6), but apoptosis was significantly increased by co-treatment of TNF with MG-132 (75.3 ± 5.1%). HTB14 (glia-like) cells did not show any significant cell death in response to even high TNF concentrations (0.1–100 ng/ml, for 24 hr), but co-treatment with TNF and MG-132 caused significant cytotoxicity (92.5 ± 3.5%; Fig. 6E).

Discussion

Conflicting views have emerged on the effects of the transcription factor NF-κB in the pathogenesis of cerebral ischemia. The present results give direct evidence that NF-κB activity is constitutively expressed in normal cerebral cortex and agree with previous data (24,28). Basal levels of NF-κB activity are normally present within neuronal nuclei in the cerebral cortex, although white matter is almost completely devoid of activated NF-κB. Our results now clearly show that focal cerebral ischemia induces a rapid and

progressive loss of cortical NF-κB IR within the ischemic core and nearby penumbra. These observations were confirmed through immunohistochemistry, Western blot analysis, and EMSA, each of which revealed similar decreases in activated NF-κB within the ischemic core and penumbra, compared to normally perfused cortex. Western blot analysis of nuclear extracts from the ischemic zone showed a significant decrease but not a complete loss of NF-κB protein. It is likely that these results are influenced by minor sampling variability, as there are no absolute criteria to distinguish ischemic core and penumbra from nonischemic tissues in the microdissection and preparation of nuclear extracts. Although variability is reduced by dissecting a minimal area of ischemic cortex, we cannot exclude the possibility that some nonischemic tissues with higher levels of NF-κB might have contributed to this signal. Thus, these results may well *underestimate* the magnitude of cerebral ischemia–induced NF-κB losses.

We have previously reported that permanent cerebral ischemia induces a rapid (maximum by 6 hr) and significant up-regulation of TNF expression within the ischemic cortex (33). The up-regulation of TNF expression and significant decrease in NF-κB activity within the zone of cortical ischemia precedes the development of apoptotic cell death (maximum about 24–48 hr). We obtained independent evidence indicating that the decline in activated NF-κB can sensitize brain cells to the cytotoxicity of TNF. When NF-κB activity is inhibited in neuronal-like (HTB 11) and glial-like (HTB14) cell cultures by addition of MG-132, these cells are exquisitely sensitized to the cytotoxic effects of TNF. Thus, a loss of NF-κB activity in the context of increased TNF expression worsens cellular damage by exacerbating cell death in both neuronal and glial cell populations. Further support for this mechanism is given by the recent data of Behl and colleagues, who showed that NF-κB levels are constitutively increased in neurons resistant to oxidative cell death and that inhibition of NF-κB activity reverses this resistance and potentiates cell death (19). Thus, in the context of either increased TNF production or enhanced oxidative damage, both of which occur in the pathogenesis of cerebral ischemia, the loss of NF-κB predisposes cells to death, thereby contributing to TNF-mediated brain damage.

While these observations do reveal a mechanism of increased cytotoxicity mediated by TNF during cerebral ischemia, they do not identify the nature of the signal that accounts for the loss of NF-κB activity in the ischemic brain. NF-κB can regulate TNF gene expression in some cells, and paradoxically, TNF can activate NF-κB by stimulating the proteolytic breakdown of IκBα (reviewed in ref. 7). Recent evidence suggests, however, that NF-κB activation is not required for TNF gene expression (45). In agreement with this observation, we observed that the loss of NF-κB activity in the ischemic brain precedes increased expression of TNF. The increase in NF-κB activity within 5 days after the onset of MCAO has been observed by others (46), and interestingly, in our experiments the kinetics and the anatomical region of increased NF-κB activity correlated with the expression of bFGF IR in astrocytes. bFGF expression may be important in the healing phases of ischemic injury by stimulating neuronal survival and sprouting, angiogenesis, and astrogliosis (47). Thus, it is now interesting to consider that delayed expression of NF-κB might participate in the regulation of late bFGF gene expression, whereas early losses of NF-κB can sensitize to TNF cytotoxicity.

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