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# Tumor necrosis factors α and β protect neurons against amyloid β-peptide toxicity: Evidence for involvement of a κB-binding factor and attenuation of peroxide and $Ca^{2+}$ accumulation

(Alzheimer disease/cell death/confocal laser scanning microscopy/fura-2 imaging/reactive oxygen species)

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ABSTRACT **In Alzheimer disease (AD) the amyloid β-peptide (Aβ) accumulates in plaques in the brain. Aβ can be neurotoxic by a mechanism involving induction of reactive oxygen species (ROS) and elevation of intracellular free calcium levels ($[Ca^{2+}]_i$). In light of evidence for an inflammatory response in the brain in AD and reports of increased levels of tumor necrosis factor (TNF) in AD brain we tested the hypothesis that TNFs affect neuronal vulnerability to Aβ. Aβ-(25–35) and Aβ-(1–40) induced neuronal degeneration in a concentration- and time-dependent manner. Pretreatment of cultures for 24 hr with TNF-β or TNF-α resulted in significant attenuation of Aβ-induced neuronal degeneration. Accumulation of peroxides induced in neurons by Aβ was significantly attenuated in TNF-pretreated cultures, and TNFs protected neurons against iron toxicity, suggesting that TNFs induce antioxidant pathways. The $[Ca^{2+}]_i$ response to glutamate (quantified by fura-2 imaging) was markedly potentiated in neurons exposed to Aβ, and this action of Aβ was suppressed in cultures pretreated with TNFs. Electrophoretic mobility-shift assays demonstrated an induction of a κB-binding activity in hippocampal cells exposed to TNFs. Exposure of cultures to IκB (MAD3) antisense oligonucleotides, a manipulation designed to induce NF-κB, mimicked the protection by TNFs. These data suggest that TNFs protect hippocampal neurons against Aβ toxicity by suppressing accumulation of ROS and $Ca^{2+}$ and that κB-dependent transcription is sufficient to mediate these effects. A modulatory role for TNF in the neurodegenerative process in AD is proposed.**

Several lines of study have led to the hypothesis that an inflammatory reaction is involved in the pathogenesis of Alzheimer disease (AD) (1). Recently, amyloid β-peptide (Aβ) was found to cooperate with interferon γ in the activation of cultured microglia, resulting in elevation of tumor necrosis factor α (TNF-α) release (2). Reactive glia are found in proximity to amyloid plaques; TNF-α is elevated in these glia (3), as are other cytokines such as interleukin 1 (IL-1) and S100β (4). It has been proposed that these factors are involved in maturation of the plaque or propagation of the pathology to surrounding tissue. For instance, IL-1 elevates expression of the β-amyloid precursor protein in endothelial cells (5) and induces the amyloid-promoting factor antichymotrypsin in astrocytes (6), and S100β may stimulate the aberrant neuritic growth in plaques (7, 8).

We recently found that TNF-α and a related cytokine, TNF-β, protect primary neurons from damage induced by glucose deprivation and glutamate (9) and protect primary astrocytes from acidosis (10). TNF-α and TNF-β (also known as lymphotoxin α) bind the same set of receptors, which are members of a family of related cell-surface molecules (11). TNF-α also facilitates axon elongation through injured nerve (12), suggesting that elevation of TNF may promote regeneration of injured fiber tracts. Insults such as ischemia (13) and traumatic brain injury (14, 15) elevate TNF-α levels in brain, suggesting that TNFs play a role in the brain's defense against neuronal damage and that the elevation of TNF-α in AD may reflect a compensatory response. While serum levels of TNF-α have been reported to be elevated in AD (16), other studies have found the levels to be unchanged or even decreased (17, 18). Such discrepancies argue that any role TNF plays in AD is not essential for progression of the disease, suggesting that its elevation may be incidental—perhaps as a reaction to the incipient damage.

Reactive oxygen species (ROS) have been implicated in many aspects of aging and in neurodegenerative diseases, including AD (19, 20). The neurotoxicity of Aβ appears to involve ROS; Aβ generates free radicals in solution (21) and induces peroxide accumulation in cultured neuroblastoma cells (22) and primary hippocampal neurons (23), and Aβ toxicity can be blocked by antioxidants (21–23). Predictably, Aβ also induces NF-κB (22), a transcription factor that can be activated posttranslationally by oxidative stress (24). A prototypical member of the Rel family of κB-binding transcription factors (25), NF-κB is also one of the most ubiquitously conserved components of TNF signaling. In addition, the genes for TNF-α and TNF-β are themselves activated by NF-κB. Therefore, it is possible that tissues damaged by an oxidative attack from Aβ respond with an increase in the levels of cytokines that offer protection against the insult. We tested this model by assaying the effects of TNFs on the toxicity of Aβ.

## MATERIALS AND METHODS

**Hippocampal Cell Culture and Analysis of Neuronal Survival.** Dissociated cell cultures of hippocampus were established from 18-day-old rat embryos according to procedures detailed previously (26). Similar procedures were used for mouse (C57BL/6) cultures. All experiments were done in 6- to 10-day-old cultures. Human recombinant TNF-α and TNF-β were purchased from PeproTech (Rocky Hill, NJ). Two different synthetic Aβ peptides of human sequence (Bachem)

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Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer disease; DCF, 2,7-dichlorofluorescein; EMSA, electrophoretic mobility-shift assay; ROS, reactive oxygen species; TNF, tumor necrosis factor; $[Ca^{2+}]_i$, intracellular $[Ca^{2+}]$; AO, antisense oligonucleotide.

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were used: Aβ-(1–40) (lot ZK600) and Aβ-(25–35) (lot ZL650). The toxicity profiles of these peptides were characterized in our previous studies (23, 27). Control peptides with reverse amino acid sequences [i.e., Aβ-(35–25) and Aβ-(40–1)] were generous gifts from Athena Neurosciences, Inc. Neuronal survival was quantified as described (26, 28).

**Measurement of Cellular Peroxides and Intracellular Free Calcium Levels ($[Ca^{2+}]_i$).** Relative levels of cellular peroxides were quantified by confocal laser microscope image analysis of cultured cells loaded with 2,7-dichlorofluorescein diacetate as detailed previously (23, 27). Procedures for quantifying $[Ca^{2+}]_i$ in individual neurons by radiometric imaging of the $Ca^{2+}$ indicator dye fura-2 are detailed in our past studies (26, 28, 29). Values represent the average $[Ca^{2+}]_i$ in the neuronal cell body.

**Electrophoretic Mobility-Shift Assay (EMSA) of κB-Binding Activity.** Nuclear extracts were prepared by the method of Ostrowski *et al.* (30). This procedure yielded 15–30 μg of nuclear protein from ≈8 × $10^6$ cells. EMSA was performed with a commercial kit (GIBCO) according to the manufacturer's instructions. Five micrograms of nuclear extract was incubated with a $^{32}P$-labeled DNA sequence containing a tandem repeat of an NF-κB binding site (boldface):

5′-GATCCAAG**GGGACT**TTCCATGGATCCAAG**GGGACT**TTCCATG-3′
3′- GTTC**CCCTGA**AAGGTACCTAGGTTC**CCCTGA**AAGGTACCTAG-5′

The DNA–protein complexes were resolved by nondenaturing low ionic strength TBE (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3)/PAGE. The gel was dried and exposed for autoradiography.

**IκB Antisense Oligonucleotides (AOs).** Experiments with MAD3 AOs were performed in cultures established from mice due to the availability of sequence data. Oligonucleotides were synthesized by Oligos Etc. (Guilford, CT) or IDT (Coralville, IA). AO against mouse MAD3 was 5′-TGGCTGAAACAT-GGC-3′; its mismatch control was 5′-TAGTTGGAACAC-GGC-3′. Oligonucleotide sequences were compared to all vertebrate GenBank sequences and no significant matches were found other than mouse MAD3.

## RESULTS

**TNFs Protect Hippocampal Neurons Against Aβ Toxicity.** Exposure of hippocampal cell cultures to Aβ-(25–35) resulted in a concentration- and time-dependent reduction in neuronal survival (Fig. 1*A*). Aβ-(1–40) also caused a progressive reduction in neuronal survival, while control peptides did not significantly reduce neuronal survival during a 3-day exposure period (Fig. 1). Based on our previous data with excitotoxic and metabolic insults (9), we tested whether TNFs would protect hippocampal neurons against Aβ toxicity. Cultures were pretreated for 24 hr with TNF-α or TNF-β (100 ng/ml) and then exposed to Aβ-(25–35) or Aβ-(1–40) [control cultures received Aβ-(35–25)]. Neuronal degeneration induced by 50 μM Aβ-(25–35) or 20 μM Aβ-(1–40) was significantly reduced in cultures pretreated with either TNF-α or TNF-β (Fig. 1*C*). TNF-α and TNF-β alone had no significant effect on basal level of neuronal survival during a 72-hr treatment (data not shown).

**TNFs Suppress Aβ-Induced Accumulation of Cellular Peroxides and Protect Neurons Against Oxidative Injury.** Cells were loaded with a compound that is converted to the fluorescent dye 2,7-dichlorofluorescein (DCF) upon interaction with peroxides. Aβ-(25–35) and Aβ-(1–40) each induced a 5-fold increase in DCF fluorescence within 4 hr (Fig. 2*A*). The Aβ-induced increases in neuronal DCF fluorescence were significantly attenuated in cultures pretreated with either TNF-α or TNF-β. TNFs were ineffective in suppressing Aβ-induced peroxide accumulation when added at the time of or after exposure to Aβ (data not shown). Aβ appeared to induce an increase in DCF fluorescence in all regions of neurons including cell bodies and neurites (Fig. 2*B*).

To determine whether TNFs increase the resistance of neurons to oxidation rather than interfere with a specific effect of Aβ, we assayed the ability of TNFs to protect neurons against iron. Addition of $FeSO_4$ (10 μM) to hippocampal cultures resulted in rapid neuronal death, with fewer than 10% of the neurons surviving a 6-hr exposure. In contrast, 50–60% of the neurons survived exposure to $FeSO_4$ in cultures pretreated for 48 hr with either TNF-α or TNF-β at 100 ng/ml (control cultures, 98% ± 2% survival; $FeSO_4$, 8% ± 3%; TNF-α and $FeSO_4$, 52% ± 6%; TNF-β and $FeSO_4$, 57% ± 7%; mean ± SEM; $n$ = 4 cultures per condition). Iron-induced neuronal death was significantly lower in cultures pretreated with TNF-α or TNF-β compared to control cultures ($P < 0.01$; ANOVA with Scheffe's post-hoc test).

**TNFs Attenuate the Potentiation by Aβ of Glutamate-Induced Toxicity and $[Ca^{2+}]_i$ Increase.** Excitotoxicity appears to play a role in Aβ toxicity (29, 31), which may explain the selective vulnerability of neurons to Aβ. Neuronal death was significantly greater in cultures exposed to the combination of Aβ plus glutamate compared to cultures exposed to Aβ or glutamate alone (Fig. 3*A*). In contrast, the combined neurotoxicity of Aβ plus glutamate was largely blocked in cultures pretreated with TNFs (Fig. 3*A*). Measurements of $[Ca^{2+}]_i$

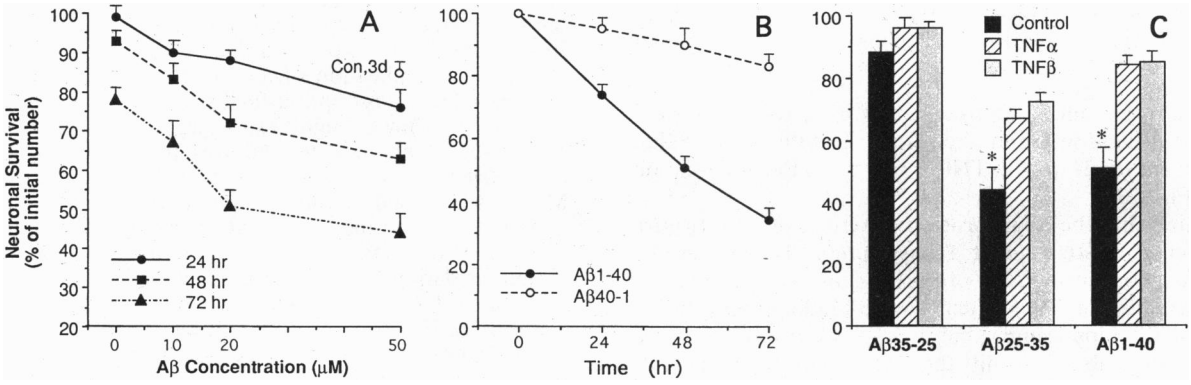


FIG. 1. TNFs protect against Aβ neurotoxicity. (*A*) Cultures were exposed to the indicated concentrations of Aβ-(25–35) and neuronal survival was monitored at 24-hr intervals during a 3-day exposure period. (*B*) Cultures were exposed to 20 μM Aβ-(1–40) or the control peptide Aβ-(40–1), and neuronal survival was monitored at 24-hr intervals during a 3-day exposure period. (*C*) Cultures were pretreated for 24 hr with saline (control and Aβ), TNF-α (100 ng/ml), or TNF-β (100 ng/ml). Cultures were then exposed to 50 μM control peptide [Aβ-(35–25)], 50 μM Aβ-(25–35), or 20 μM Aβ-(1–40) for 48 hr, and neuronal survival during the period of exposure to Aβ was determined. Values represent means and SEM of determinations made in four separate cultures. *, $P < 0.005$ compared to control value in cultures exposed to Aβ-(35–25) and $P < 0.01$ compared to corresponding values for cultures pretreated with TNF-α and TNF-β.

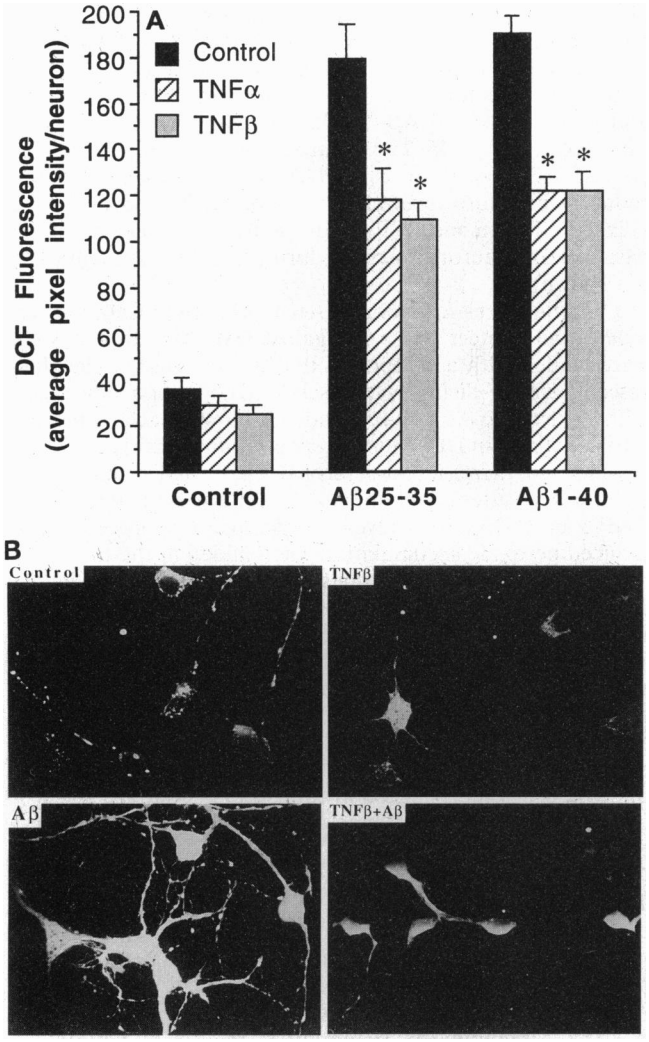


FIG. 2. TNFs suppress Aβ-induced accumulation of peroxides. (*A*) Cultures were pretreated for 24 hr with saline (Control), TNF-α (100 ng/ml), or TNF-β (100 ng/ml). Cultures were then exposed to vehicle (Control), 50 μM Aβ-(25–35), or 20 μM Aβ-(1–40) for 4 hr and relative levels of cellular peroxides were determined. Values represent means and SEM of determinations made in three separate cultures (17–23 neurons). *, $P < 0.005$ compared to cultures not exposed to Aβ and $P < 0.01$ compared to corresponding control value. (*B*) Confocal laser scanning microscopy of cellular peroxides in cultured hippocampal neurons. Images of DCF fluorescence in hippocampal neurons are shown. Cultures were pretreated for 24 hr with saline (control and Aβ) or TNF-β (100 ng/ml) and then exposed to vehicle or 50 μM Aβ for 3 hr.

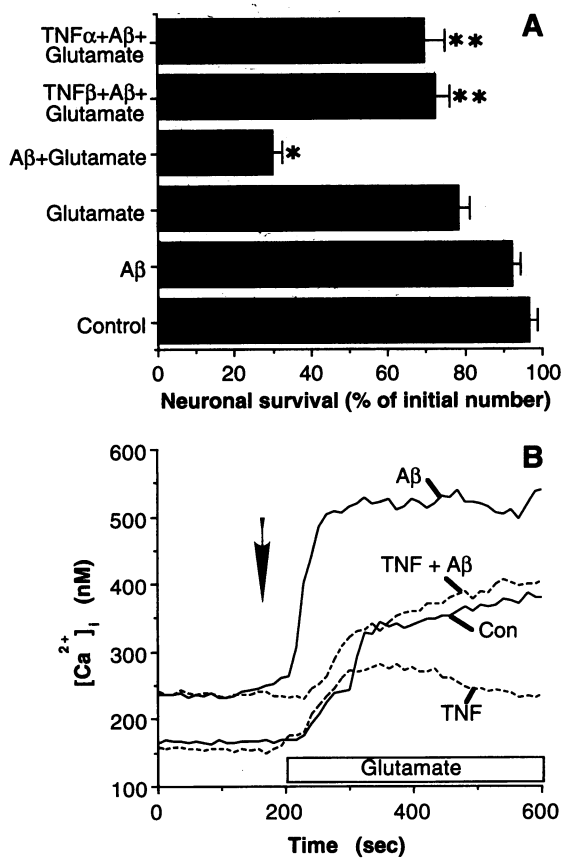


FIG. 3. TNFs attenuate Aβ-induced potentiation of glutamate effects. (*A*) Cultures were exposed to the indicated agents: TNF-α or TNF-β, 100 ng/ml (added to cultures 24 hr prior to exposure to Aβ); 20 μM Aβ-(25–35) (added to cultures 6 hr before exposure to glutamate); 10 μM glutamate. Neuronal survival was assessed 14 hr after exposure to glutamate. Values represent means and SEM of determinations made in four separate cultures. *, $P < 0.01$ compared to corresponding values for control, Aβ, or glutamate alone. **, $P < 0.01$ compared to value for cultures exposed to Aβ and glutamate (ANOVA with Scheffe's post-hoc test for pairwise comparisons). (*B*) Cultures were pretreated for 24 hr with saline (control and Aβ) or TNF-β (100 ng/ml) and then exposed to vehicle or 50 μM Aβ for 3 hr. The $[Ca^{2+}]_i$ was determined at 8-sec intervals before and after exposure to 50 μM glutamate (arrow indicates time of addition of glutamate). Values represent means of 28–50 neurons in three or four separate cultures.

responses to glutamate showed that $[Ca^{2+}]_i$ responses were enhanced in neurons pretreated for 3 hr with Aβ and that pretreatment for 24 hr with TNF-β prevented the potentiation by Aβ (Fig. 3*B*).

**Evidence That the Neuroprotective Actions of TNF Involve Activation of κB-Dependent Transcription.** To address the mechanism by which TNFs protect against Aβ toxicity, we tested the ability of TNFs to activate κB-binding transcription factors in rat hippocampal cultures. Nuclear extracts were assayed for the ability to shift the electrophoretic mobility of DNA containing the κB enhancer element. Extracts from cultures treated with TNF-α or TNF-β showed an increase over controls in their ability to generate a specific protein–DNA complex (Fig. 4*A*). To address the role of κB-dependent transcription in the protection of TNFs against Aβ toxicity, we sought a means to induce κB-binding factors directly. NF-κB is sequestered in the cytosol in a latent state by various inhibitory proteins collectively termed IκB (32). Induction of the active state involves dissociation and degradation of IκB. In previous studies, we found that reduction of a specific IκB (MAD3) by application of an AO results in activation of a κB-dependent transcription factor (33). Neurons exposed to MAD3 AO showed significantly less death in response to Aβ-(25–35) than in sister cultures that received a control oligonucleotide or no oligonucleotide (Fig. 4*B*). Furthermore, MAD3 AO caused a striking inhibition of the effects of Aβ on glutamate-evoked increases in $[Ca^{2+}]_i$ (Fig. 4*C*). The protective effects of MAD3 AO apparently included the ability to inhibit oxidation, as a 24-hr pretreatment also reduced the increase in DCF fluorescence induced by Aβ. After a 5-hr exposure to 20 μM Aβ-(25–35), the average pixel intensities of DCF fluorescent neurons in cultures were as follows: not treated with oligonucleotide, 73.61 ± 5.99; with MAD3 AO, 43.86 ± 0.63; mismatch oligonucleotide, 72.25 ± 4.55 (mean ± SEM; $n = 3$ cultures per condition).

## DISCUSSION

Our data suggest that TNF is an injury response factor that may offer protection against neural insults associated with AD. The

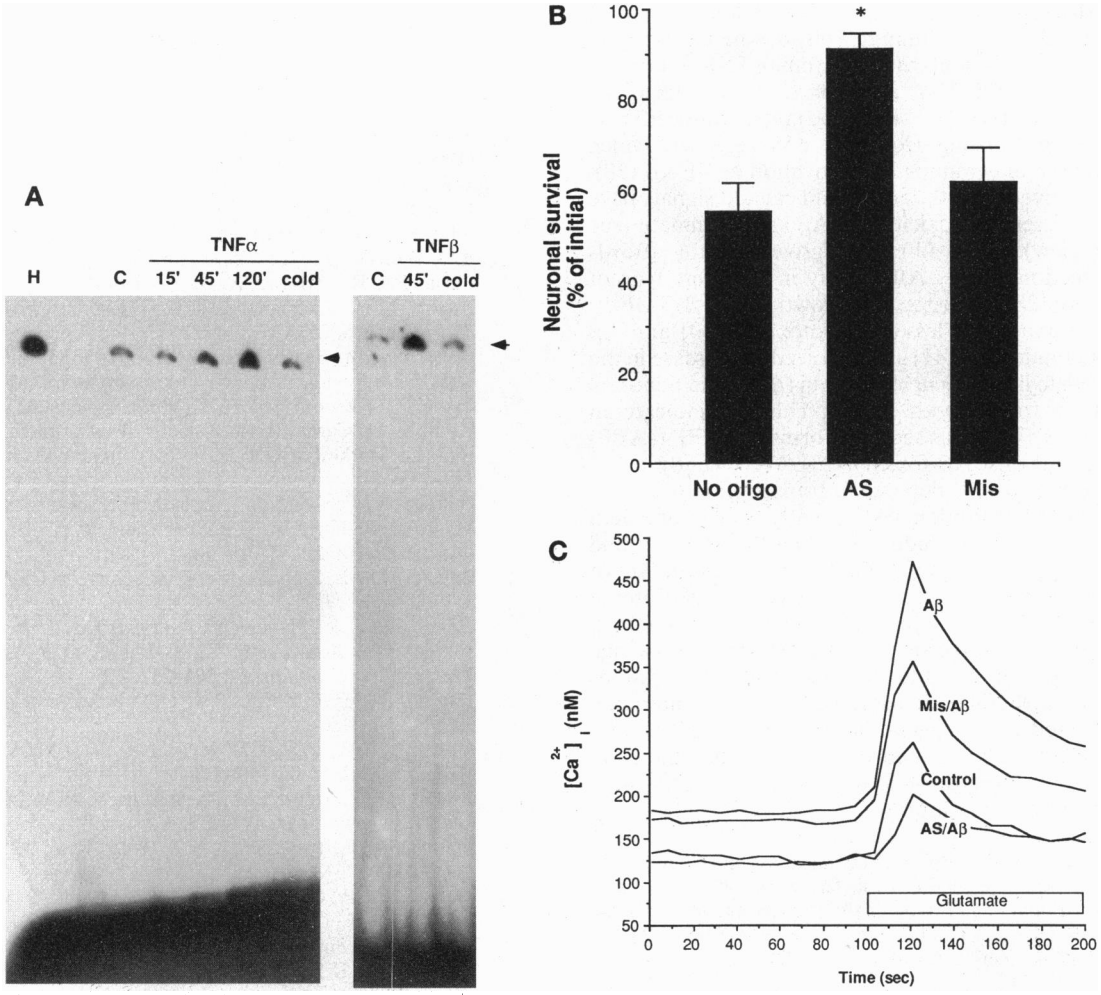


FIG. 4. Evidence that NF-κB mediates the neuroprotective and $[Ca^{2+}]_i$-stabilizing actions of TNFs. (*A*) EMSA on nuclear extracts (5 μg of protein per lane) from untreated cultures (lanes C) or cultures that had been exposed to TNF-α or TNF-β at 100 ng/ml for the indicated times. A nuclear extract from HeLa cells (lane H) was analyzed as a positive control. Both TNF-α and TNF-β induced a protein–κB complex (arrowheads). Specificity of the complexes was demonstrated by competition of the same samples with an excess of unlabeled DNA (cold). (*B*) Primary mouse hippocampal cultures were exposed to no oligonucleotide (No oligo), 20 μM MAD3 AO (AS), or 20 μM mismatch control oligonucleotide (Mis) for 24 hr. Neuronal viability was determined after an additional 24-hr exposure to 20 μM Aβ-(25–35). Values are means and SEM ($n = 3$). *, $P < 0.05$ compared to corresponding value for mismatch control or no oligonucleotide. (*C*) Mouse hippocampal cultures were treated for 24 hr with no oligonucleotide (Aβ), 20 μM MAD3 AO (AS/Aβ), or 20 μM mismatch control oligonucleotide (Mis/Aβ). Cultures were then exposed to 20 μM Aβ-(25–35) for 6 hr. The $[Ca^{2+}]_i$ was determined at 8-sec intervals before and after exposure to 5 μM glutamate (at 100 sec). The glutamate response was also recorded in cells that had not been exposed to oligonucleotide or Aβ (Control). Values represent means of 70–100 neurons in three or four separate cultures.

treatment of primary hippocampal cultures with TNF-α or TNF-β afforded substantial protection against the neurotoxicity of Aβ. The enhanced survival of TNF-treated neurons was associated with attenuation of mechanisms implicated in Aβ toxicity; Aβ induced lower levels of ROS and dampened responses of $[Ca^{2+}]_i$ to glutamate in the TNF-treated cultures. Moreover, TNF-α and TNF-β induced a transcription factor that binds the κB enhancer element; this transcriptional machinery may explain the protection of TNFs against Aβ. These findings imply that TNFs may be initially part of a compensatory response that occurs to combat neurodegenerative conditions.

The similarities in the abilities of TNF-α and TNF-β to protect neurons against various insults probably reflect convergence of their signaling pathways. One component of cytokine signaling that appears to be shared by TNF-α and TNF-β is the activation of NF-κB. The mimickry of TNFs' neuroprotection by disinhibition of NF-κB indicates that the transcriptional sequelae initiated are sufficient to provide neuroprotection. The connection of NF-κB to the control of genes that increase cellular antioxidant capacity (24) provides a reasonable explanation for the neuroprotective activity of the TNFs. TNF-α increases the expression of manganese superoxide dismutase in neural cells (34), probably through κB enhancer sites in its promoter. In the present study evoked ROS were dampened by IκB antisense, which is presumed to act through gene regulation. NF-κB and/or other κB-dependent transcription factors may act to coordinate antioxidant expression with the oxidative load of a cell (24). This type of feedback mechanism may even utilize peptide factors such as the TNFs in order to more diffusely propagate a signal more stable than the ROS themselves.

Some studies have documented neurotoxicity of TNF-α (35, 36). In at least one case (35), the toxicity was apparently mediated by microglia in coculture with the neurons; the other study utilized cultures established from fetal human cerebral cortex, which also tend to have large numbers of glia. The rodent cultures used in the present study are relatively deficient in glia. Therefore, one explanation for the apparent discrepancies is that TNFs are directly beneficial to neuronal viability but that TNF-α can evoke responses from glia that are harmful to neurons in coculture. Species differences may also

contribute to discrepancies between studies. Gelbard *et al.* (36) treated human cultures with human TNF-$\alpha$, whereas we have used human TNF-$\alpha$ in rodent cultures. Human TNF-$\alpha$ does not activate the rodent p75 TNF receptor (37); therefore, the neuroprotective effects of TNF-$\alpha$ may be mediated specifically by signaling events arising from the p55 receptor, which include production of ceramide and activation of NF-$\kappa$B (38).

Other glial-derived growth factors and related signals have been shown to lessen the toxicities of A$\beta$ and glutamate (see ref. 39 for review). Basic fibroblast growth factor affords complete protection against A$\beta$ toxicity in the same type of cells studied here (28). Transforming growth factor $\beta$ (TGF-$\beta$) has also been reported to lessen excitotoxicity (40) and A$\beta$ toxicity (41). Galindo *et al.* (41) also reported an increase in the expression of $\beta$-amyloid precursor protein ($\beta$APP) in neuronal and glial cultures treated with TGF-$\beta$. This may indicate an indirect action for TGF-$\beta$, as secreted forms of $\beta$APP (sAPP) can also protect against A$\beta$ toxicity (23). We recently found that sAPP also induces $\kappa$B-dependent transcription (42), further linking this transcription system with neuroprotection. Several growth factors are induced in glia by A$\beta$ itself and accumulate in neuritic plaques (43). Accumulation in AD of other glial-derived neuroprotective proteins such as protease nexin I and $\alpha$1-antichymotrypsin may similarly reflect an overreaction of the glia–neuron axis as it attempts to manage the neurodegeneration. This and other systemic considerations limit the potential therapeutic usefulness of TNFs; however, the data presented here suggest that pharmacological mimickry of the effects of TNFs in neurons—particularly induction of $\kappa$B-dependent transcription—may offer clues for alleviating A$\beta$-induced neurodegeneration.

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