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Tumor Necrosis Factor and Reactive Oxygen Species Cooperative Cytotoxicity Is Mediated via Inhibition of NF-κB

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Abstract

Background: Tumor necrosis factor alpha (TNFα) plays a key role in pathogenesis of brain injury. However, TNFα exhibits no cytotoxicity in primary cultures of brain cells. This discrepancy suggests that other pathogenic stimuli that exist in the setting of brain injury precipitate TNFα cytotoxicity. The hypothesis was tested that reactive oxygen species (ROS), that are released early after brain injury, act synergistically with TNFα in causing cell death.
Materials and Methods: Cultured human and rat brain capillary endothelial cells (RBEC), and cortical astrocytes were treated with TNFα alone or together with different doses of H_2O_2, and apoptotic cell death and DNA fragmentation were measured by means of 3′-OH-terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and Hoechst fluorescence assay, respectively. The effect of H_2O_2 on TNFα-induced activation of nuclear factor kappa B (NF-κB) was measured by Western blots of cytoplasmic and nuclear extracts of RBEC using anti-inhibitor of NF-κB (IκB) and anti-p65 subunit of NF-κB antibodies. Nuclear translocation of NF-κB was investigated by immunofluorescent staining of RBEC with anti-p65 antibodies.
Results: TNFα alone had no cytotoxic effect in brain endothelial cells and astrocytes at concentrations up to100 ng/ml. Co-treatment with 5–10 μM of H_2O_2 caused a two-fold increase in the number of apoptotic cells 24 hr later. Similar doses (1–3 μM) of H_2O_2 initiated early DNA fragmentation. H_2O_2 inhibited TNFα-induced accumulation of p65 in the nucleus, although it had no effect on degradation of the IκB in cytoplasm. Immunostaining confirmed that H_2O_2 inhibited p65 transport to the nucleus.
Conclusions: Reactive oxygen species could act synergistically with TNFα in causing cytotoxicity via inhibition of a cytoprotective branch of TNFα signaling pathways, which starts with NF-κB activation.

Introduction

The pleiotropic cytokine tumor necrosis factor alpha (TNFα) exerts biological activity in CNS (1–4). TNFα effects in brain parenchyma are shown to play a key role in brain injury (5–8). High TNFα levels have been detected in brain trauma (9–11) and ischemia (12–15). Neutralization of TNFα by TNFα-binding protein had a protective effect against focal ischemia (16,17) and trauma (18) and an inhibitor of TNFα synthesis, dexanabinol has a protective effect in closed head injury (CHI) and MCAO (19,20).

However, in vitro studies demonstrate that TNFα is not cytotoxic in brain cells. It even causes protection of cultured neurons (21–23). Cultured cortical astrocytes and brain endothelial cells treated with TNFα for 48 hr exhibit no signs of apoptosis (24). The discrepancy between observations of a TNFα pathogenic

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function in animal models of brain injury and its lack of cytotoxic effect on brain cells in vitro suggests that other pathogenic stimuli contribute to TNFα cytotoxicity in the setting of brain injury.

Reactive oxygen species are among the most toxic mediators released early after brain injury. The brain is extremely vulnerable to oxidative damage (25). We have shown that the synthetic spin-trap antioxidant from the nitroxide family, Tempol, improved recovery and protected the blood–brain barrier in a rat model of CHI (26). Similar protection was found after CHI in heat-acclimated rats, in which the endogenous antioxidants have been shown to be elevated (27). On the other hand, TNFα levels and activity were not affected in Tempol-treated or heat-acclimated animals (28), suggesting that ROS could alter TNFα signaling rather than TNFα synthesis and thus precipitate TNFα cytotoxicity. Similarly, the same spin-trap molecule, was used in studies of bacterial and cultured mammalian cells and was shown to provide cytoprotection from the toxicity induced by TNFα (29). Transcription of many pro-inflammatory, immune, and apoptotic genes, which are induced by TNFα, is dependent on activation of nuclear factor kappa B (NF-κB). Each step of NF-κB activation and DNA binding is redox sensitive (30). Taken together, these observations suggest that the point of intersection of TNFα and ROS, which both accompany brain insults, could be NF-κB. The present study was designed to test this hypothesis. We demonstrate here that sublethal doses of H_2O_2 abrogate natural resistance of different types of brain cells to TNFα by inhibiting TNFα-induced activation of NF-κB.

Materials and Methods

Human brain capillary endothelial cells (HBEC) cultures have been previously described (31). Rat brain capillary endothelial cells (RBEC) were prepared from adult WKY rat brains as for human cultures except that fetal bovine serum was substituted for human serum and 90 μg/ml heparin was added to the medium. The purity of the both HBEC and RBEC was >95% as determined by positive immunostaining for von Willebrand factor (Factor VIII), and angiotensin-converting enzyme, incorporation of acetylated low-density lipoprotein, and by negative staining for glial cells (GFAP, galactocerebroside, ED-2), muscle cells (α-actin) and pericytes (tropomyosin). Human brain capillary endothelial cells were at passage 4. Rat brain capillary endothelial cells were at passages 7–12 and were seeded from four different batches of brain tissue. Cortical astrocyte cultures were established from 3-day-old Sprague-Dawley rats as described (24).

Visualization and quantitation of apoptotic cells was performed by means of 3′-OH-terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) using "In Situ Cell Death Detection Kit (POD)" (Boehringer Mannheim, Germany). Human brain capillary endothelial cells and RBEC were treated with 15 ng/ml human TNFα (Endogen, Woburn, MA, USA) and 20 ng/ml rat TNFα (Chemicon International Inc., Temecula, CA, USA), respectively, or/and with different doses of H_2O_2 for 24 hr, fixed with 4% paraformaldehyde for 30 min and stained according to the manufacture's protocol. The samples were analyzed with a Zeiss Axiovert 100 light microscope (20× objective). Digitized images of 15 microscopic fields per each experimental condition were generated using a digital CCD Camera C4742-95-12 (Hamamatsu) and Zeiss AxioVersion 2 software. The same microscope and camera settings were used for all samples. The number of apoptotic cells within each image was determined by means of Scion Image (NIH Image for PC) computer program. Briefly, background was subtracted from each image and each image was transformed into a binary image, which permitted measurement of the area occupied by all of the cells in the image (area T). All the images were then reversed to multi-gray mode, and the average optical density of nonstained cells was measured for all images acquired. The value of the mean density of nonstained cells was subtracted from all the images and the remaining areas of higher density (area A) (these were positively stained apoptotic nuclei) were again thresholded to binary images and measured.

Percentage of apoptotic cells was calculated as follows:

$$\%\ \text{apoptotic nuclei} = \frac{rA}{\mathrm{T}} * 100$$

where: A is the sum of the TUNEL-positive areas in the image, and T is the sum of the areas

occupied by all cells in the image.

$$r = \frac{\text{size of the cell}}{\text{average size of the nucleus}};$$

$$r = 3.25 \pm 0.75 \ (n = 15)$$

Quantitation of DNA fragmentation was performed by means of a fluorescent, cell-permeable, DNA-binding dye, Hoechst 33342 as previously described (24). Hoechst fluorescence upon binding to DNA is inversely proportional to the degree of DNA fragmentation in the cells undergoing apoptosis (32). Briefly, TNFα/H_2O_2-treated astrocytes or RBEC, plated in 96 well microtiter plates were incubated with 25 μM Hoechst 33342 (Molecular Probes, Eugene, OR, USA) in PBS added at 100 μl/well for 45 min at 37°C. Cell fluorescence was measured using a CytoFluor 4000 fluorescent plate reader (PerSeptive Biosystems, Framingham, MA, USA) at excitation/emission wavelengths of 360/460 nm. Background fluorescence was measured on each plate and subtracted. Each data point was a mean of fluorescence readings of eight wells (variability was less than 30%). The percentage of apoptotic cells was calculated from Hoechst fluorescence by means of the following formula:

$$\%\ \text{apoptotic cells} = \frac{F\ \max - F}{F\ \max - F\ \min} * 100\%$$

where F_{max} is fluorescence of untreated healthy control cultures, F_{min} is fluorescence of cells treated with the cytotoxic alkylating agent methyl iodide, and F is fluorescence of unknown sample.

Immunofluorescent Staining for p65 Subunit of NF-κB

Endothelial cells were treated with 20 ng/ml TNFα and with or without various doses of H_2O_2 for 25 min. Cells were fixed with ethanol for 2 min and then with 3.7% formaldehyde for 5 min and immunostained with rabbit polyclonal antibody directed against the p65 subunit of NF-κB (Santa Cruz, cat. #sc109) or with mouse monoclonal antibody against the activated form of p65 (Boehringer-Manheim Cat. #1697838) both antibodies were at 1:50 dilution according to Kaltschmidt et al. (69). Detection was performed with anti-rabbit and anti-mouse corresponding biotinylated secondary antibody, followed by addition of streptavidin-Cy3. Digitized images of the fluorescent cells were generated using the same microscope and camera as for TUNEL experiments (40× objective).

Preparation of Cytosolic and Nuclear Extracts

Rat brain capillary endothelial cells were grown to confluency in 60-mm dishes. Rat TNFα (Chemicon International, Temecula, CA, USA) was added to the cells at 20 ng/ml with or without 2 μM H_2O_2. At the indicated times, cells were placed on ice, washed twice with PBS, and then scraped off into 800-μl PBS containing protease inhibitor cocktail (Boehringer Mannheim), phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4), and 1 mM dithiothreitol (DTT). Cells were pelleted in a microcentrifuge for 1 min at 2,500 rpm, resuspended in 5 volumes (~100 μl/dish) of a low salt buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, pH = 7.9) and incubated for 15 min on ice. At the end of incubation NP-40 was added to the lysates at final concentration 0.1%. Samples were vigorously vortexed for 20 sec and centrifuged at 11,000 rpm for 1 min. Cytoplasmic fraction was transferred to a new Eppendorf tube, and frozen at −70°C. The pellet was resuspended in 30–50 μl/dish high salt buffer B (20 mM HEPES, 400 mM NaCl, 50 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% (w/v) glycerol, pH = 7.9). The samples were shaken at a high speed for 30 min and then microcentrifuged at 14,000 rpm for 5 min. The supernatant was frozen at −70°C. Prior to freezing, a 2-μl aliquot from each cytosolic and nuclear extract was taken for protein determination (Bio-Rad Laboratories, Hercules, CA, USA). All reagents were ice-cold, and all procedures were performed on ice.

Western Blots for NF-κB Studies

All the buffers, 4–12% Tris-glycine gradient mini-gels, nitrocellulose membranes, and electrophoresis equipment were from Novex (San Diego, CA, USA). Cytosolic or nuclear extracts were boiled in equal volumes of loading buffer/1 mM DTT for 3 min and loaded on a gel at 10 μg protein/lane for determination of p65 concentrations in nuclear extracts, and at 15 μg protein/lane for IκB determination in cytosolic fractions. Separated proteins were transferred to nitrocellulose membrane. Immunoblotting was performed as previously described (33). Anti-IκBα and anti-p65 rabbit polyclonal antibodies were from Santa Cruz Biotechnology (SC-372 and SC-203, respectively).

Electrophoretic mobility shift assays (EMSA) were performed by using the Gel Shift Assay System (Promega) according to the manufacturer's instruction. Nuclear extracts were prepared from untreated control cells, and cells treated with TNFα alone or together with 5 μM H_2O_2. The NF-κB consensus oligonucleotide (AGTTGAGGGGACTTTCCCAGGC) was end-labeled using [γ-^{32}P]ATP. The reaction mixture contained 4 μl 5× gel shift binding buffer, 1 μl of ^{32}P labeled NF-κB (15,000 cpm) consensus oligonucleotide probe, 10 μg of nuclear extract in a total volume of 20 μl. The reaction was incubated at room temperature for 20 min. After incubation, the samples were loaded on 4% nondenaturing polyacrylamide gel and were electrophoresed at 150 volts for 3 hr. The gel was dried and autoradiograph was developed. After the autoradiography, the bands were cut out, counted, and the specific radioactivity associated with each band was calculated.

Statistical analysis was carried out by one-way ANOVA followed by Dunnett's test and by one-way ANOVA for repeated measures followed by Turkey test using SigmaStat Software (p values $< .05$ were considered statistically significant). All graph data are presented as Mean ± SD.

Results

TNFα and H_2O_2 Have a Synergistic Effect on Induction of Apoptosis

It has previously been shown that human TNFα was not cytotoxic for cultured HBEC at doses of 15 ng/ml (250 EU/ml), although this dose caused cell activation (34). Similarly in RBEC, while causing activation of ICAM-1 adhesion ligand, TNFα exhibited no cytotoxicity at the dose of 20 ng/ml (24). Thus, these doses of TNFα were adopted for current investigation. To test the hypothesis that the combination of TNFα and ROS will have a synergistic effect on cell viability, HBEC were treated with a combination of TNFα (15 ng/ml) and H_2O_2 (100 μM) for 4 hr. Cell viability was monitored under phase contrast field. When added separately, TNFα and H_2O_2 caused no morphological changes (Fig. 1B and C) when compared to control cultures (Fig. 1A), addition of both agonists resulted in cell death (Fig. 1D).

To further investigate the effect of H_2O_2 cells were treated with lower doses of H_2O_2 (5 and 10 μM for HBEC and RBEC, respectively) in the presence of TNFα for 24 hr and the number of apoptotic cells was quantitated with TUNEL assay. The results are presented in Figure 2A (HBEC) and Figure 2B (RBEC). The top panel of each figure presents photomicrographs of endothelial cultures after treatment. The bottom pannel presents binary images of corresponding photomicrographs with highlighted TUNEL-positive areas. TNFα alone for 24 hr remained healthy, with few TUNEL-positive cells (photomicrographs a and binary images d in both Fig. 2A and B). Similarly, H_2O_2 alone had no effect on cell viability (photomicrograph b and binary image e in both Fig. 2A and B). Addition of H_2O_2 together with TNFα resulted in the appearance of many TUNEL-positive cells exhibiting apoptotic morphology (shrinking of the cytoplasm, chromatin condensation, pyknosis) (photomicrograph c and binary image f in both Fig. 2A and B). Results of quantitation of percentage of TUNEL-positive nuclei with ScionImage program are presented in Table 1. It demonstrates that endothelial cell cultures treated with both TNFα and H_2O_2 exhibited two times more apoptotic cells than control cultures or cultures treated with TNFα or H_2O_2 alone ($p < .05$).

To study the synergistic effect of TNFα and H_2O_2 in more detail, we have used the Hoechst assay, which is apparently more sensitive indicator of DNA fragmentation (32). Besides endothelial cells, cortical astrocytes also have been studied. Cells were treated with TNFα in combination with various doses (0–10 μM) of H_2O_2. Because DNA fragmentation occurs early in the course of apoptosis, the cultures were evaluated 15–16 hr after addition of TNFα and of H_2O_2. Lower doses of H_2O_2 (1–3 μM) were required to initiate DNA fragmentation measured in this assay compared to TUNEL assay as cells need a longer time to undergo full-blown apoptosis. Rat brain capillary endothelial cells were more sensitive to TNFα/H_2O_2 treatment than astrocytes. Addition of as little as 0.3–1 μM of H_2O_2 together with 10 ng/ml TNFα resulted in apoptosis of the majority of the RBEC (Fig. 3A), whereas 1–3 μM H_2O_2 caused the same effect in astrocytes (Fig. 3B).

H_2O_2 Inhibits TNFα-induced Activation of NF-κB

Transcription of many TNFα-activated genes depends on the transcription factor, NF-κB. However, NF-κB activation could be altered by

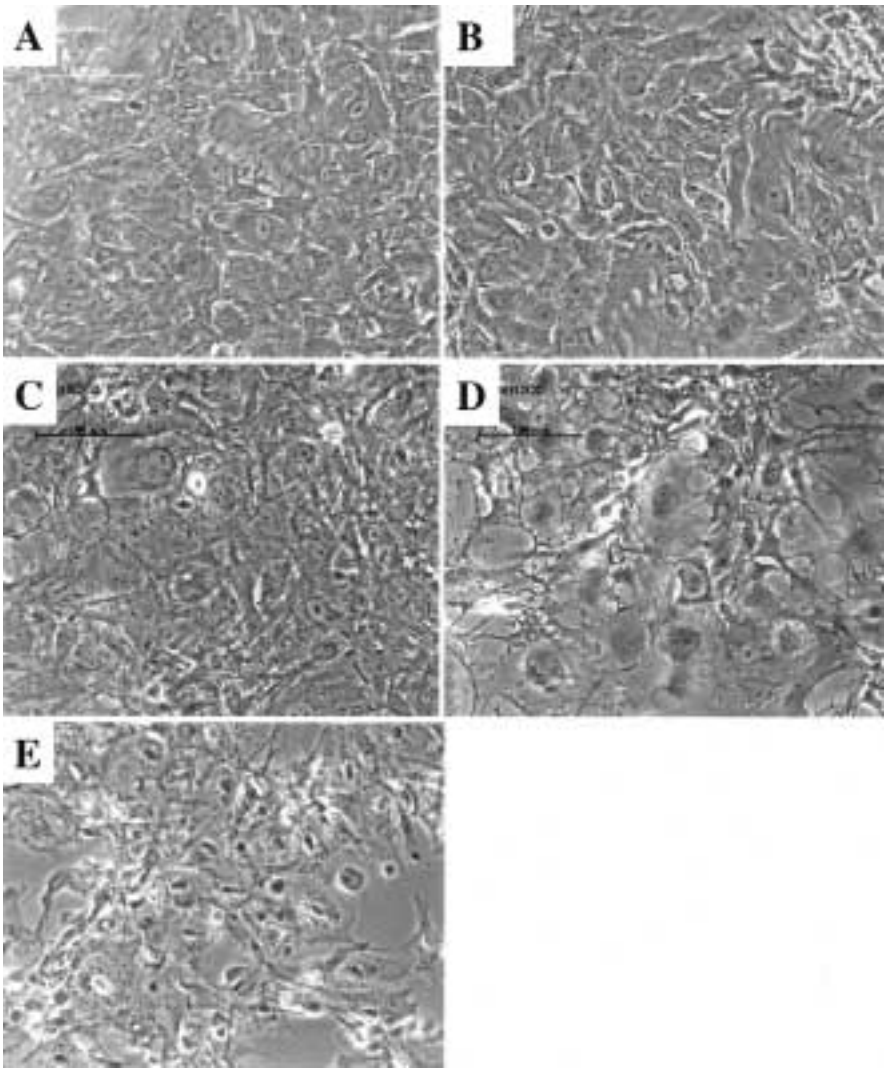

Fig. 1. Synergistic effect of tumor necrosis factor alpha and H_2O_2 on induction of apoptosis. Human brain capillary endothelial cells were treated with a combination of TNFα (15 ng/ml) and H_2O_2 (100 μM) for 24 hr. Cell viability was monitored under phase contrast field (objective 20×). (A) Untreated cells; (B) TNFα alone; (C) H_2O_2 alone; (D) TNFα and H_2O_2 together; (E) NF-κB inhibitor BAY11-7082.

ROS and antioxidants. Thus, we hypothesized that H_2O_2 might interfere with TNFα-induced NF-κB activation precipitating cell death. To test this hypothesis, we have studied NF-κB activation in RBEC. Cells were treated with 20 ng/ml TNFα for various times, and then IκB and p65 levels were determined in cytoplasmic and nuclear extracts, respectively, by means of Western blotting and ScionImage analysis as described in Materials and Methods. The results of these experiments are presented in Figure 4A–D. IκB degradation in cytoplasm began as early as 10 min after TNFα addition; at 20 min IκB levels in cytoplasm dropped to 22.5 ± 6.2% (mean ± SD; $n = 4$) of baseline levels and remained that low for another 10 min. At 1 hr after TNFα addition, IκB levels returned to about 70% of control values (Fig. 4A and B). Addition of 2 μM H_2O_2 had no significant effect on IκB degradation. IκB degradation had almost identical kinetics in the absence or presence of H_2O_2. Addition of 2 μM H_2O_2 alone without TNFα caused no degradation of IκB. At 20 min, IκB levels were 109.8 ± 0.05% control (mean ± SD; $n = 2$). There was a constitutive p65 presence in the nucleus of brain cells. In all experiments ($n = 5$), antibody identified two bands. We suggest that one of the bands is a phosphorylated form of p65. Accumulation of p65 subunit in the nucleus

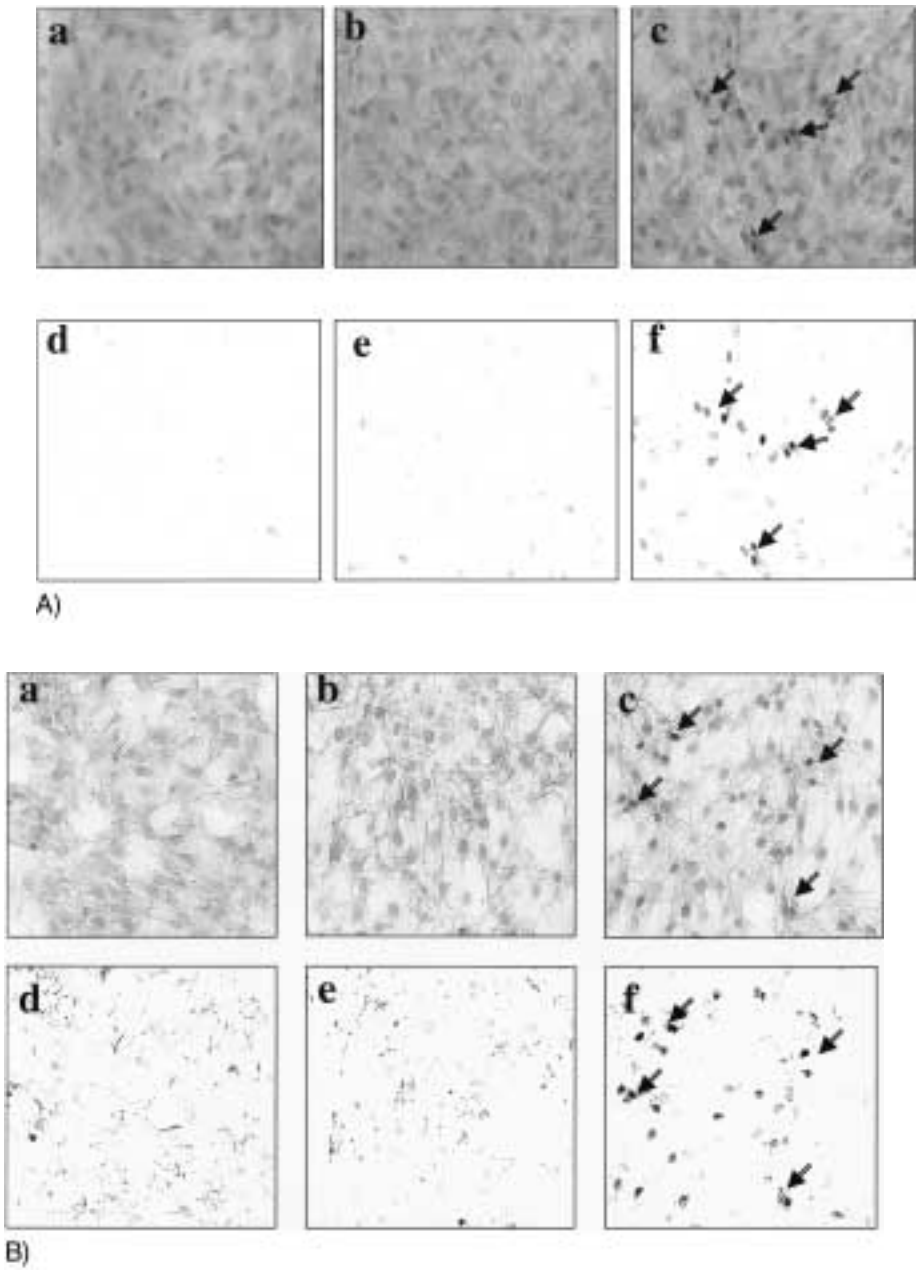

Fig. 2. Synergistic effect of tumor necrosis factor alpha (TNFα) and H_2O_2 on the number of TUNEL-positive cells. (A) Human brain capillary endothelial cells, and (B) rat brain capillary endothelial cells were treated with either TNFα (photomicrographs a and d) or H_2O_2 (photomicrographs b and e) or with both agonists (photomicrographs c and f) for 24 hr and apoptotic nuclei were visualized with TUNEL assay as described in Materials and Methods. Photomicrographs are representatives of 15 images captured for each condition. The top row of panels (A) and (B) (photomicrographs a, b, and c) are phase-contrast images, the bottom row of panels (A) and (B) (photomicrographs d, e, and f) are corresponding binary images. Apoptotic nuclei are indicated with arrows.

paralleled degradation of IκB in the cytoplasm and peaked at 20 min after TNFα addition (153.0 $\pm$ 25.8% baseline; mean $\pm$ SD; $n = 5$) (Fig. 4C and D). Addition of 2 μM H_2O_2 to the cells simultaneously with TNFα completely inhibited p65 translocation to the nucleus: measured p65 levels at 20 min were 109.5 $\pm$ 21.1% baseline ($p < 0.05$ vs. TNFα). Addition of 2 μM H_2O_2 alone without TNFα resulted in no significant increase of p65 levels in the nucleus. At 20 min, p65 levels were 110.6 $\pm$ 5.7% control (mean $\pm$ SD; $n = 3$).

Table 1. Synergistic effect of TNFα and H_2O_2 on induction of apoptosis and percentage of TUNEL-positive nuclei

	Treatment	TNFα H_2O_2	TNFα none	none H_2O_2	none none
RBEC 1	% apoptotic cells	27.7 ± 8.0*	14.8 ± 11.9	15.8 ± 9.4	14.8 ± 8.8
RBEC 2	% apoptotic cells	31.3 ± 11.8*	17.4 ± 6.1	12.7 ± 4.7	14.8 ± 6.6
HBEC	% apoptotic cells	30.8 ± 9.1*	7.2 ± 3.3	4.2 ± 2.5	2.6 ± 1.5

Rat brain capillary endothelial cells and human brain capillary endothelial cells were treated with either TNFα or H_2O_2 or with both agonists for 24 hr and apoptotic nuclei were stained with TUNEL assay and quantitated by means of ScionImage software as described in Materials and Methods. Each data point represents percentage of TUNEL-positive nuclei per a microscopic field (mean ± SD of 15 microscopic fields). The summaries of the results of three separate experiments (two in RBEC and one in HBEC) are presented. Statistical comparison between synergistic treatment with TNFα and H_2O_2 and separate treatments was performed by means of one-way ANOVA followed by Dunnett's test. Data marked with * statistically differ from all other groups (p values $< .05$).

The results of Western blots were confirmed with electrophoretic mobility shift assay. Nuclear extracts of untreated HBEC cells had little DNA-binding activity. Drastic upregulation of DNA-binding activity was caused by TNFα alone. However, addition of 5 μM H_2O_2 together with TNFα inhibited DNA binding by 40% (Fig. 4E and F).

H_2O_2 Inhibits Nuclear Transport of p65 Subunit of NF-κB

Two subunits comprise NF-κB, which belong to Rel family of proteins. The most frequently found NF-κB heterodimer is p65/p50. Interaction of the subunits with the inhibitor of NF-κB (IκB) masks the nuclear translocation sequence of NF-κB and retains the heterodimer in cytoplasm. Upon activation with TNFα and other agonists, IκB gets phosphorylated by a specific kinase, ubiquitinated, and subsequently degraded by proteasome peptidases (35). Removal of IκB-alpha uncovers the nuclear localization signals of subunits of NF-κB, allowing the complex to enter the nucleus, bind to DNA, and affect gene expression. The results of Western blots presented in Figure 4 demonstrate that, although TNFα-induced IκB degradation was not affected by H_2O_2 (Fig. 4A and B), and should result in release of a free p65/p55 heterodimer in cytoplasm, the p65 subunit did not appear in the nucleus (Fig. 4C and D), suggesting that transport of p65 from cytoplasm to the nucleus was compromised. To confirm this assumption, RBEC were treated with 20 ng/ml TNF-α or with 5 μM H_2O_2 or with both agonists for 30 min and then fixed and immunostained with anti-p65 antibodies. In control, untreated cells (Fig. 5A) and in the cells treated with H_2O_2 alone (Fig. 5B), immunofluorescence was spread over cytoplasm and was not found in the nucleus. Treatment with TNFα caused redistribution of fluorescence with maximal signal coming from the nucleus (arrows) (Fig. 5C). Although there was some background fluorescence in the nuclei of control and H_2O_2-treated cells (Fig. 5A and B), it had an appearance of small granules, whereas in TNF-treated cells (Fig. 5C), the whole nucleus was highlighted. Addition of TNFα together with H_2O_2 resulted in no accumulation of fluorescence in the nucleus, and most of the bright spots are localized in perinuclear forming a dense circle (Fig. 5D). This pattern was somewhat different from that of control cells, and was consistently observed in all captured images. Similar results were obtained in HBEC using an antibody directed against an activated form of p65 (data not shown).

Inhibitor of NF-κB Causes Apoptosis

To investigate the role NF-κB in survival of endothelial cells, HBEC were incubated with different doses (1–10 μM) of the NF-κB inhibitor BAY 11-7082 (E-3-[4-methylphenylsulfonyl]-2-propenenitrile) (Biomol Research Laboratories, Inc. Plymouth Meeting, PA, USA). Cell morphology was analyzed 4 hr later with a Zeiss Axiovert 100 light microscope (20× objective) and the images were captured as described in Materials and Methods. At a dose as low as 1 μM, the inhibitor caused apoptosis (Fig. 1E).

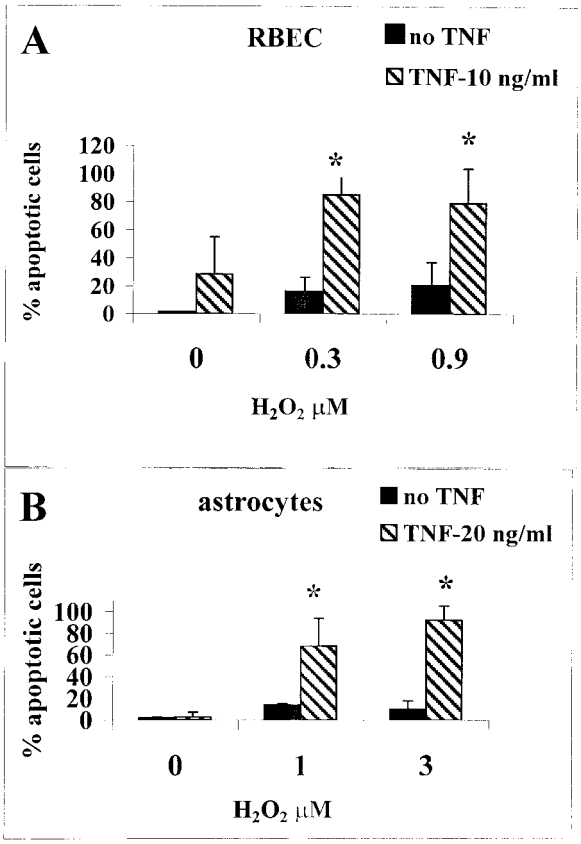

Fig. 3. Synergistic effect of tumor necrosis factor alpha (TNFα) and H_2O_2 on induction of DNA fragmentation. (A) Rat brain capillary endothelial cells, and (B) astrocytes were grown to confluency in 96 well plates. Cells were incubated with various doses of H_2O_2 in the presence or absence of TNFα for 16 hr. At the end of the incubation, cells were stained with Hoechst 33342 and decrease of fluorescence dependent on DNA fragmentation was quantitated by a fluorescent plate reader as described in Materials and Methods. Each measurement was done in six wells and averaged. The bar graphs represent mean ± SD of three experiments performed in different cell cultures. "*" Marks significant difference between the treatments with and without TNF ($p < .05$).

Discussion

Ischemic and traumatic brain injury are accompanied by oxidative stress (36–38) and release of proinflammatory cytokines, TNFα and IL-1β (8,39). Although these pathogenic reactions have been extensively studied, there is no consensus of opinion on mechanisms of their action and interaction. Oxidants have been shown to stimulate signaling pathways usually triggered by growth factors (activation of protein tyrosine kinases and phosphatases, PKC and mitogen-activated kinases, phospholipases Cγ and A_2 and Ca^{2+} (40). Similarly, it has been thought that TNFα also induces release of ROS (mainly H_2O_2) in mitochondria (41) and through NADPH oxidase (42) and then ROS act as messengers in TNFα signaling pathways leading to NF-κB-dependent transcription of pro-inflammatory genes (43–45) and to cell death (46). However, although H_2O_2 activates NF-κB in some cell types, it fails to do so in human endothelial cells (47,48), in lymphoblastoid (49), and in monocytic cell lines (50). Moreover, since the first observations of the anti-apoptotic effects of NF-κB have been published (51–53), the role of NF-κB in cell death has been revised. Much evidence has emerged that demonstrates that NF-κB activates transcription of protective genes in different types of cells (54), including brain cells (55,56) rather than causing cell death. Furthermore, inhibition of NF-κB sensitizes neurons to cytotoxic effects of amyloid beta (57), and activation of NF-κB promotes neuronal survival (58,59). These observations suggest an alternative mechanism for interaction between TNFα and ROS in induction of cell death.

In this work, we present evidence supporting the hypothesis that ROS cooperate with TNFα and induce cell death via inhibition of NF-κB. For the first time, we demonstrate that low doses of H_2O_2, which are not capable of causing cell death on their own, synergize with TNFα and unmask TNFα cytotoxicity in cultured brain endothelial cells and astrocytes. This conclusion is based on the results of morphological studies and TUNEL staining for apoptotic cells, as well as on quantitation of DNA fragmentation with Hoechst 33342, which is a cell-permeable DNA-binding fluorescent dye. Hoechst fluorescence is inversely proportional to the degree of DNA fragmentation (60). A synergistic effect of TNFα and H_2O_2 was demonstrated in both assays. When treated with TNFα alone for 24 hr, HBEC, RBEC, and astrocytes showed a low rate of apoptosis, most probably caused by culture conditions. However, addition of low doses of H_2O_2, together with TNFα, results in early DNA fragmentation followed by appearance of TUNEL-positive apoptotic cells. Interestingly, in our preliminary experiments, we used a cell-impermeable fluorescent dye, ethidium homodimer, to assess cell viability. Healthy cells exclude ethidium, but those with a damaged plasma membrane, generally necrotic

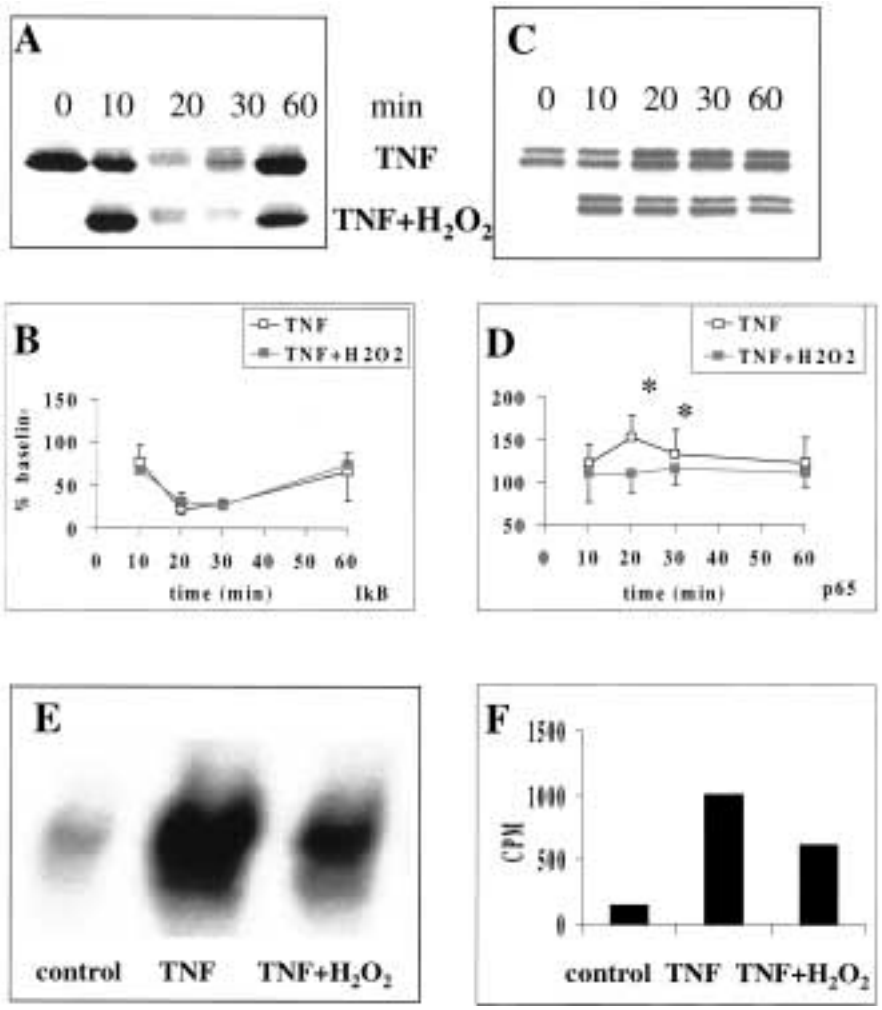

Fig. 4. H_2O_2 inhibits tumor necrosis factor alpha (TNFα)-induced activation of NF-κB. Rat brain capillary endothelial cells were activated with TNFα in the absence or presence of 2–5 μM H_2O_2 for indicated times (0–60 min). At the end of each incubation, cytoplasmic and nuclear extracts were prepared as described in Materials and Methods. Extracts were subjected to SDS-PAGE and immunobloted with antibodies directed either against IκB (cytocolic extracts, panels A and B) or against p65 subunit of NF-κB (nuclear extracts, panels C and D). (A) and (B) Representative results of Western blots. (B) and (D) Results of densitometry analysis of protein bands. Each data point represents mean ± SD of four to five experiments. "*" Marks significant difference between the treatments with and without H_2O_2 (ANOVA for repetitive measurements; $p < .05$). (E) and (F) Representative of electrophoretic mobility shift assays (EMSA) ($n = 3$). Nuclear extracts were prepared from untreated cells (control), or cells treated with TNFα alone (TNF) or together with 5 μM H_2O_2 (TNF + H_2O_2) and EMSA was performed as described in Materials and Methods. (F) The specific radioactivity associated with each band is presented.

cells, accumulate the dye and become highly fluorescent. In these experiments, doses of H_2O_2 required to produce membrane permeability (ethidium assay) were higher (10–20 μM depending on the culture; data not shown) than those needed to cause DNA fragmentation (1–5 μM) in the presence of the same dose of TNFα. This dichotomy fits the definition of necrosis and apoptosis well. Necrosis is an uncontrolled degenerative phenomenon invariably caused by noxious stimuli and is the result of irreversible failure of membrane function. In contrast, apoptosis is a death process that involves a series of well-organized events that require active cell participation, and is primarily caused by physiological stimuli. Previous observations showing that low doses of ROS induce apoptosis, whereas necrosis occurs in cells exposed to higher doses of ROS (61–63) are in accordance with our findings.

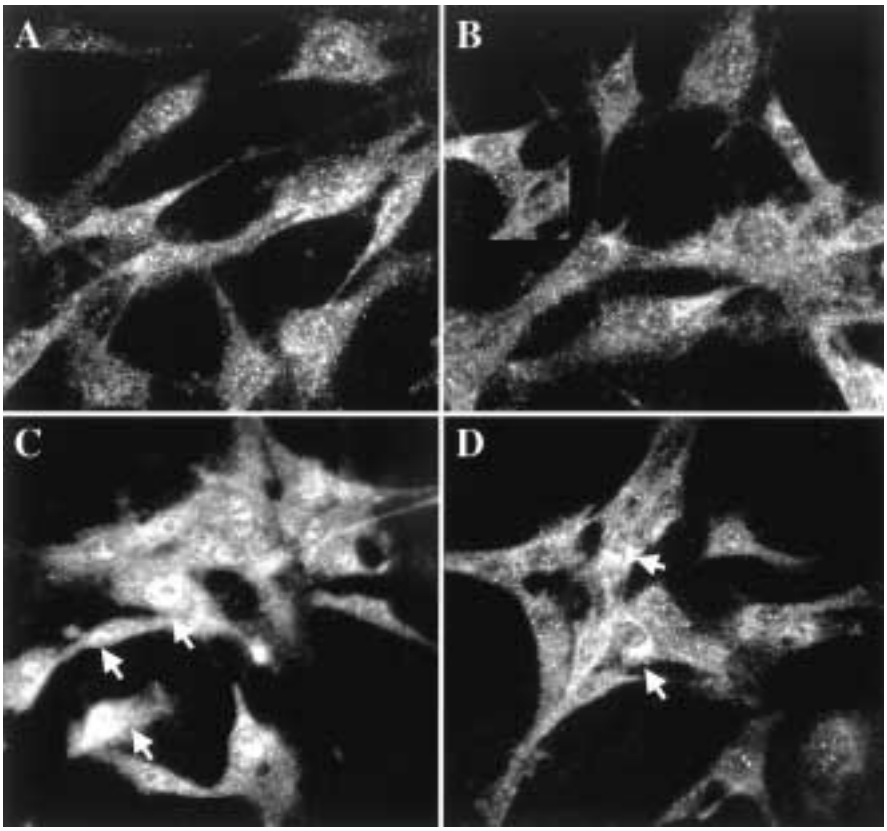

Fig. 5. H_2O_2 inhibits tumor necrosis factor alpha (TNFα)-induced nuclear translocation of NF-κB. (A) rat brain capillary endothelial cells untreated and those treated with (B) H_2O_2 or with (C) TNFα alone or with (D) both agonists for 30 min, were fixed and immunostained with anti-p65 antibody as described in Materials and Methods. Arrows denote accumulation of immunofluorescence in the nucleus in TNFα-treated cells (C) and in perinuclear area in cells treated with TNFα and H_2O_2 (D).

Chemical reactivity and redox potentials of ROS range from reducing to oxidizing: superoxide anion radical can be reduced to H_2O_2 by superoxide dismutase, the latter can be further reduced by the Fenton reaction with iron to the hydroxyl radical, which is capable of oxidizing nucleic acids, lipids, and proteins. Employment of specific scavengers for different types of ROS has demonstrated that some but not all of these species activate NF-κB (64). It has been demonstrated that all the steps of NF-κB activation (IκB phosphorylation and degradation, p65/p55 nuclear translocation, and DNA binding) are redox-sensitive (65–68). Our data suggests that low doses of ROS have no effect on the steps involved in degradation of IκB but alter TNFα-induced nuclear translocation of at least the p65 subunit of NF-κB. Quantitation of IκB levels in TNFα-treated RBEC demonstrated almost complete disappearance of the inhibitor from the cytoplasm at 20 min; IκB gene itself has an NF-κB binding site, so it is quickly resynthesized (69). By 60 min ~60% of IκB protein could be detected in TNFα-stimulated RBEC. These kinetics of IκB degradation and resynthesis did not change in the presence of H_2O_2, which means that free NF-κB heterodimer is released in the cytoplasm and should be transported to the nucleus. However, we were unable to detect increased levels of p65 in the nucleus in the cells treated with TNFα and H_2O_2, although TNFα alone triggered a 1.5-fold increase of p65 levels in the nucleus. This result was confirmed by gel-shift assay and immunostaining experiments, which demonstrated that TNFα failed to induce translocation of the p65 subunit to the nucleus in the presence of H_2O_2 and instead caused accumulation of the p65 in the perinuclear area. Similarly, high doses of amyloid beta peptide, known to elicit production of ROS, have been shown to inhibit nuclear transport of p65 (57,70).

Our data strongly suggest that H_2O_2 precipitates TNFα cytotoxicity by inhibiting transcription of the NF-κB-dependent protective genes. However, other mechanisms are not excluded by these studies. Thus treatment of the cells with low concentrations of H_2O_2 induces activation of caspases, cysteine proteases that constitute part of apoptotic machinery (63). In addition, TNFα-induced activation of sphingomyelinase and consequent release of ceramide, a phospholipid messenger implicated in apoptosis, could be prevented by antioxidants and stimulated by H_2O_2 in astrocytes (71). In our studies, we have shown that physiological doses of C-2 ceramide failed to induce apoptosis in cultured astrocytes and RBEC but higher doses were apoptotic (33). Another possibility is that ROS by interfering with ceramide metabolism allow higher intracellular levels of ceramide and cause cell death.

There is evidence that NF-κB is constitutively expressed in neuronal cells and mediates their resistance to different types of stress (55,72,73), and that neurons from mice lacking the p50 subunit of NF-κB are more vulnerable to excitotoxic stress (74). Our Western blot data demonstrate constitutive presence of p65 in the nucleus of brain microvascular cells and inhibition of this NF-κB activity in HBEC cultures by low doses of NF-κB inhibitor BAY11-7082 resulted in rapid apoptosis. Our results are consistent with findings of Taglialatela et al. (76). However, observations in animals are not consistent. Thus suppression of NF-κB activity in brain by a specific inhibitor resulted in DNA fragmentation (76), but TNFα receptor knockout mice, which exhibited delayed up-regulation of NF-κB after traumatic brain injury, had a larger average lesion volume and blood brain barrier breach than wild-type animals (77). Also, p50 knockout mice tolerated ischemic injury better than wild-type animals (74). NF-κB activity was shown to increase after brain trauma (79) and in the model of transient focal ischemia 72 hr after reperfusion. (78). In the latter model and also in the model of intracerebral hemorrhage, activated NF-κB colocalized with apoptotic cells (78,80). However, in a rat model of permanent MCAO, activated NF-κB immunoreactivity decreased from basal levels already at 2 hr after onset of ischemia and remained undetectable up to 5 days (75). Interestingly, antioxidant-dependent protection against transient focal ischemia was associated with inhibition of NF-κB (81) but in global ischemia antioxidants inhibited only persistent NF-κB activity in hyppocampal CA1 neurons, whereas transient activation of NF-κB seemed to be protective (82). Overexpression of Mn-SOD in human breast cancer MCF-7 cells completely abolished TNF-mediated NF-κB activation, and caused apoptosis (83). These contradictions call for more detailed studies of NF-B activation in vivo. Time, localization, and variations in NF-κB heterodimer composition should be taken into account.

In conclusion, the data presented here suggests a new pharmacological approach to the treatment of brain injury. Instead of targeting TNFα and ROS separately, one might want to interfere with the cross-talk mechanisms of these two pathogenic pathways that modulate NF-κB dependent anti-apoptotic signaling.

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