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Original Research Communication

ROS-Production–Mediated Activation of AP-1 but Not NFκB Inhibits Glutamate-Induced HT4 Neuronal Cell Death

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ABSTRACT

Aside from their deleterious effect, reactive oxygen species (ROS) can function as small messenger molecules during physiologic processes. ROS have been shown to activate the transcription nuclear factor kappa B (NFκB) and activator protein 1 (AP-1). Exposure of HT4 neuronal cells to 10 m*M* glutamate results in cell death after 12 h. Here we show that glutamate treatment leads to an increase in ROS production and activation of AP-1, but not NFκB. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA), an activator of protein kinase C and an inducer of NFκB and AP-1, protected the cells. This protective effect was preceded by increased production of ROS compared with glutamate alone, which was accompanied by a synergistic increase in AP-1, but not NFκB activity. We used all-*trans*-retinoic acid (ATRA), overexpression of retinoic acid receptor α (RARα) and a decoy oligonucleotide inclusion assay to suppress AP-1 activity. NFκB was inhibited by using a super suppressor (IκBαΔN-transfected cells). Inhibition of AP-1, but not NFκB resulted in increased cellular vulnerability to glutamate. Inhibition of AP-1 activity was coincident with a decrease in ROS production. Thus, although ROS are significant to the cell-death effect induced by glutamate, they also activate protective pathways mediated by increasing AP-1 activity, and not that of NFκB. *Antioxid. Redox Signal.* 8, 1339–1349.

INTRODUCTION

Elevated levels of extracellular glutamate are associated with neuronal damage and degeneration in brain disorders, including epilepsy, stroke, and Parkinson's disease (4). Two defined mechanisms of glutamate cytotoxicity exist: the excitotoxicity and excitotoxicity-independent pathways. In the excitotoxicity pathway, superactivation of glutamate ionotropic receptors is followed by an increase in free intracellular calcium levels, and the generation of reactive oxygen species (ROS) in the cell (35). The excitotoxicity-independent process relies on an imbalance between oxidants and antioxidants in the cell (oxidative stress): inhibition of cystine uptake via the x_c^- cystine/glutamate antiporter system by glutamate leads to depletion in glutathione (GSH), the major intracellular antioxidant, and excessive production of ROS (28, 34).

The physiologic relevance of the cystine inhibition–oxidative stress pathway has been debated, as it requires much higher glutamate levels than the receptor-mediated excitotoxic pathway. However, 100 μ*M* glutamate is sufficient to inhibit the import of cystine, whereas in pathologic conditions such as ischemia, extracellular glutamate can reach levels exceeding 500 μ*M*. Furthermore, evidence suggests that these two pathways are activated in neuronal injuries (26, 38, 45). An established *in vitro* model system for investigating glutamate-oxidative-stress–mediated cell death is the immortalized mouse hippocampal cell line HT4, which phenotypically resembles neuronal precursor cells. Depletion in GSH and increased production of ROS in these cells are preceded by activation of c-Src and 12-Lox (15, 40).

Antioxidants protect different cell types from glutamate cytotoxicity in both pathways, which implicates the important role of ROS in glutamate-mediated neuronal cell death

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(3, 19, 27, 30, 47). 12-*O*-Tetradecanoylphorbol 13-acetate (TPA), a phorbol ester which activates protein kinase C (PKC), also protects the cells, although ROS levels remain high. The protective effect of TPA is efficient only if added in the first 4 h of glutamate treatment, which suggests that TPA blocks a relatively early step in the pathway leading to cell death (5, 45).

With oxidative stress, ROS can damage proteins, lipids, and nucleic acids, and lead to cell death (22, 48). However, it is now well established that moderate levels of ROS, especially in the form of H_2O_2, and changes in intracellular reduction–oxidation (redox) state, act as signal-transduction messengers that can lead to the activation of transcription factors and posttranslational modifications of proteins (14, 31). Nevertheless, the physiologic role of ROS-mediated signaling is far from being understood.

Two well-defined transcription factors are redox regulated: nuclear factor kappa B (NFκB) and activator protein 1 (AP-1) (41, 44). Extracellular glutamate and oxidants such as H_2O_2 are only two examples of the many stimuli that can lead to NFκB nuclear activation (12, 37). NFκB can regulate both death proteins and proteins that enhance survival, and a wide variety of *in vitro* studies have demonstrated that in neurons, the transcriptional activity of NFκB may serve different functions, depending on the cell type (3, 18).

The implications of the signaling mediated by AP-1 DNA-binding activity in neurons also are controversial. Increased AP-1 transcriptional activity is associated with neuronal cell death. Glutamate-mediated, as well as H_2O_2-mediated cell death, is preceded by the expression of the immediate-early gene *c-fos*, which can form heterodimers with *Jun* family proteins, and the resulting AP-1 complexes regulate transcription by binding to the AP-1 sequence found in many cellular genes (33, 35, 53). The question is whether increased AP-1 activity is part of a death or survival pathway. Many studies have suggested that its activation promotes neuronal cell death (2, 9, 55), but other studies have demonstrated AP-1 activity to be essential for neuronal survival (33, 51); thus, the question remains unsolved.

The objective of the current study was to explore whether ROS can regulate cell death or survival pathways through activation of NFκB and AP-1 in glutamate-induced HT4 neuronal cell death, as a model system for a glutamate-induced excitotoxicity-independent pathway.

MATERIALS AND METHODS

Materials

Sources were as follows: L-glutamic acid monosodium salt, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), all-trans retinoic acid (ATRA), propidium iodide (PI) (Sigma–Aldrich, St. Louis, MO); dichlorofluorescein (DCF), dihydroethidium (DHE) (Molecular Probes, Eugene, OR); primary antibodies for RARα (sc-551) and IκBα (sc-371) (Santa Cruz Biotechnologies, Santa Cruz, CA); secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA); luciferase reporter system (Promega Corp., San Luis Obispo, CA); ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK); developing film (Fuji Super RX, Dusseldorf, Germany).

Cell culture

Mouse hippocampal HT4 cells, kindly provided by D.E. Koshland, Jr., University of California at Berkeley, were grown in Dulbecco's Modified Eagles Medium supplemented with 10% fetal calf serum, 1x glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. Semiconfluent cells were trypsinized and seeded on a culture dish or six-well plates at a concentration of 25,000 cells/ml. After 24 h of seeding, the culture medium was replaced with fresh medium supplemented with serum and antibiotics as stated earlier, and the cells were exposed to 10 m*M* glutamate, according to a model of glutamate-induced cytotoxicity (40, 47). ATRA and TPA were dissolved to an initial concentration of 1 m*M* in dimethyl sulfoxide (DMSO) for ATRA and ethanol for TPA, and kept at −20°C. ATRA and TPA were added directly to the cell-culture medium at a final concentration of 1 μ*M*. The corresponding controls were treated with the same volume of DMSO (ATRA control) or ethanol (TPA control).

Cell viability

Loss of cell-membrane integrity was detected by using propidium iodide (PI) staining. After glutamate treatment, cells were trypsinized, centrifuged (2,500 rpm, 5 min), and resuspended in phosphate-buffered saline (PBS). Cells were filtered through a 90-μm mesh grid, stained with PI (2 μg/ml), and measured by flow cytometry (FACSort, BD) with the fluorescence setting of excitation at 488 nm and emission at 575 nm. Data were collected from 10,000 cells (40, 46).

Intracellular ROS

H_2DCF-reacting ROS (mainly peroxides) were detected by flow cytometer (47). After the different treatments, cells were trypsinized, centrifuged (2,500 rpm, 5 min), and resuspended in PBS. Then the cells were filtered through a 90-μm mesh grid and incubated with H_2DCF (25 μ*M*) for 30 min at 37°C. The fluorescence was measured with excitation at 488 nm and emission at 530 nm. Superoxide levels were detected with DHE, dissolved to an initial concentration of 10 m*M* in DMSO (43). After the different treatments, the cells were incubated with 25 μ*M* DHE in 1 ml PBS for 30 min at 37°C. The wells were washed 3 times with cold PBS; the cells were trypsinized and analyzed with flow cytometry with excitation at 488 nm and emission at 575 nm. Data were collected from 10,000 cells.

H_2DCF was used to evaluate ROS; this is more specific to hydrogen peroxide analysis because its oxidation depends on intracellular peroxidase activity in the presence of hydrogen peroxides. This probe has high reactivity to hydrogen peroxide, lipid hydroperoxide, and hydroxyl radicals and low reactivity to superoxide anions (16, 50). DHE was used to detect superoxide anions. DHE is a redox-sensitive probe and has been widely used to detect intracellular superoxide anion. It is common knowledge that the reaction between superoxide and DHE results in the formation of a two-electron oxidized

product, ethidium (E+), which binds to DNA and leads to the enhancement of fluorescence (54). In glutamate-treated cells, these probes indeed revealed different effects on ROS production, indicating measurements of different types of ROS (see Results).

Transient transfections

Plasmids: human retinoic acid receptor α (RARα) gene, cloned in pSG5 vector, was kindly provided by Paul T. van der Saag, Netherlands Institute for Developmental Biology, Utrecht, The Netherlands. To suppress NFκB activation, we used pCM4 vector, expressing a deletion mutant of human-IκBα encoding amino acids 37 to 317 (IκBαΔN) (1), kindly provided by Dean W. Ballard, Vanderbilt University School of Medicine, Nashville, TN. This mutation does not interfere with the formation of IκBα–NFκB complex, but it prevents the release of NFκB because it lacks phosphorylation sites. HT4 cells were transfected with 1 μg DNA by using lipofectamine reagent, according to the manufacturer's instructions (GIBCO BRL, Gaithersburg, MD). Protein translation was verified by Western blot analysis. The culture medium was replaced 24 h after transfection, and cells were exposed to 10 m*M* glutamate. Control cells were transfected with empty vectors by using the same amount of DNA, to rule out any interference of the transfection protocol (pSG5 for pSG5/hRARα and promoterless pEGFPN1 for IκBαΔN).

AP-1 reporter assay

We used the AP-1-Luc vector (Clontech), which contains the luciferase gene driven by the TATA box of the thymidine kinase promoter and an AP-1–dependent enhancer element. The AP-1-Luc vector was cotransfected with a vector expressing β-galactosidase (β-gal) to standardize the transfection assay. The plasmids were transfected into HT4 cells by using lipofectamine reagent, as described earlier. Luciferase activity was measured with a luminometer and standardized in relation to β-gal activity, which was assayed in 100 m*M* sodium phosphate buffer (pH 7.5) by the hydrolysis of ONPG at 37°C for 40 min and measured by absorbance at 450 nm. The ratio of luciferase to β-Gal activity was calculated as relative luciferase activity for each sample (11).

Western blots (RARα, IκBα)

Cells were trypsinized 24 h after transfection, washed 3 times with PBS, and centrifuged (2,500 rpm, 5 min). Western blot analysis was performed as previously described (42). In brief: boiling lysis buffer was added to the cell-containing pellet; protein content was measured and equalized. Sample buffer (containing SDS) was added, and the samples (40 μg protein) were subjected to SDS polyacrylamide gel electrophoresis followed by Western blot analysis. The membrane was blocked and then incubated overnight with primary antibodies at room temperature, diluted in Blotto (RARα, 1:1,000; IκBα, 1:2,000). After being washed 6 times, the membrane was incubated for 2 h at 4°C with secondary antibody diluted in Blotto (RARα, 1:10,000; IκBα, 1:20,000). Immunoreactive bands were detected with ECL Western blotting detection reagents.

Preparation of nuclear extracts

Nuclear extracts were prepared as reported by Shilo *et al*. (42) with a slight modification. Treated cells were trypsinized, washed twice with cold PBS, and centrifuged. Hypotonic buffer (1 ml) was added to the cell pellets [20 m*M* HEPES, pH 7.0, 10 m*M* KCl, 1 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol (DTT), 0.1% Triton X-100, 20% glycerol, 2 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml aprotinin and leupeptin]. Pellets were disrupted by pipetization and centrifuged at 3,000 r/min for 10 min. The pellets were resuspended in 100 μl of cold extraction buffer [20 m*M* HEPES, pH 7.0, 10 m*M* KCl, 1 m*M* $MgCl_2$, 0.5 m*M* DTT, 0.1% Triton X-100, 20% glycerol, 2 m*M* PMSF, 1 mg/ml aprotonin and leupeptin, 420 m*M* NaCl]. The samples were rotated for 30 min at 4°C and then centrifuged at 14,000 r/min for 1 h. The supernatant was collected. Protein content was measured by using Bradford reagent. Samples were kept frozen at −70°C.

Electromobility-shift assay (EMSA) analysis

Gel-shift assays were performed according to the gel-shift assay protocol from Promega Corp. A double-stranded NFκB consensus oligonucleotide, 5′-AGTTGAGGGGACTTTCCCAGGC-3′, and the AP-1 (c-*Jun*) consensus oligonucleotide, 5′-CGCTTGAT GAGTCAGCCGGAA-3′, were labeled with ^{32}P. For each reaction, 5 μl of nuclease-free water was added to 1 to 2 μg of nuclear extract, which was then incubated with 2 μl of 5× binding buffer for 10 min (for AP-1, $MgCl_2$ was added to the 5× binding buffer to a final concentration of 10 m*M*). Then 1 μl of the ^{32}P-labeled oligonucleotide, prepared as described previously (10), was added, and the samples were incubated at room temperature for 20 min. Dye-free gel-loading solution was added (1 μl per sample), and samples were then separated by 6% nondenaturing acrylamide gel electrophoresis. The gel was dried, exposed to a phosphoimaging screen, and then visualized with a phosphoimager (FLA5OO; Fujifilm, Japan). For specificity, 1 μl of unlabeled consensus oligonucleotide (100x) was used to compete with the labeled probe.

Decoy oligonucleotide

HT4 cells were transfected with 0.1 μg AP-1 (c-*Jun*) double-stranded consensus oligonucleotide (see sequence earlier) by using lipofectamine reagent, according to the manufacturer's instructions (GIBCO BRL) and as reported by Polytarchou *et al*. (29), with slight modifications. Cells were incubated with the decoy oligonucleotide in 1 ml OptiMEM. After 6 h, 1 ml of Dulbecco's Modified Eagles Medium supplemented with 20% fetal calf serum, 1x glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml) was added for an additional 6 h. The culture medium was replaced, and cells were exposed to 10 m*M* glutamate for 15 h. Control cells were transfected with the scrambled double-stranded oligonucleotide: 5′-GGTACATTGCGACGTACGAGC-3′, by using the same amount of DNA, to rule out any interference of the transfection protocol.

Statistical analysis

Comparisons between two groups were performed with Student's *t* test. For multiple groups, data were analyzed by

analysis of variance (ANOVA). Bars represent average ± SD of three experiments. Differences were considered significant at probability levels of $p < 0.05$ by using the Fisher's protected least-significant-difference method. Statistical analysis was performed by using the statistical computer program, SPSS version 11 (SPSS Inc., Chicago, IL).

RESULTS

TPA protects against glutamate-induced cell death, promotes ROS production and nuclear activity of NFκB and AP-1

Exposure of HT4 cells to glutamate resulted in cell death 12 to 15 h later. TPA, a phorbol ester that activates PKC, protected the cells when added 5 min before the glutamate treatment (Fig. 1A). The protective effect was observed even 24 h after glutamate treatment (Fig. 1B). To study the earlier events in glutamate-induced cell death and the protective effect of TPA, we measured H_2DCF-reacting ROS levels, which are more specific to H_2O_2 and peroxides, 6 h after glutamate treatment (before any decrease in cell viability was detected). Glutamate-mediated ROS production is maximal 6 h after treatment (47). Whereas TPA itself induced only a small increase in ROS production measured by H_2DCF oxidation in the cells, it significantly potentiated the glutamate-induced ROS production (Fig. 1C). Surprisingly, in the presence of such high ROS levels, a protective effect was observed (Fig. 1A and B). The use of DHE to evaluate ROS production, which is more specific to superoxide, showed a decreased level after glutamate treatment (Fig. 1C). These data indicate that the steady-state levels of superoxide versus hydrogen peroxide production shifted toward the latter. As NFκB and AP-1 are well-defined redox-regulated transcription factors, we studied their role in TPA-mediated ROS production and

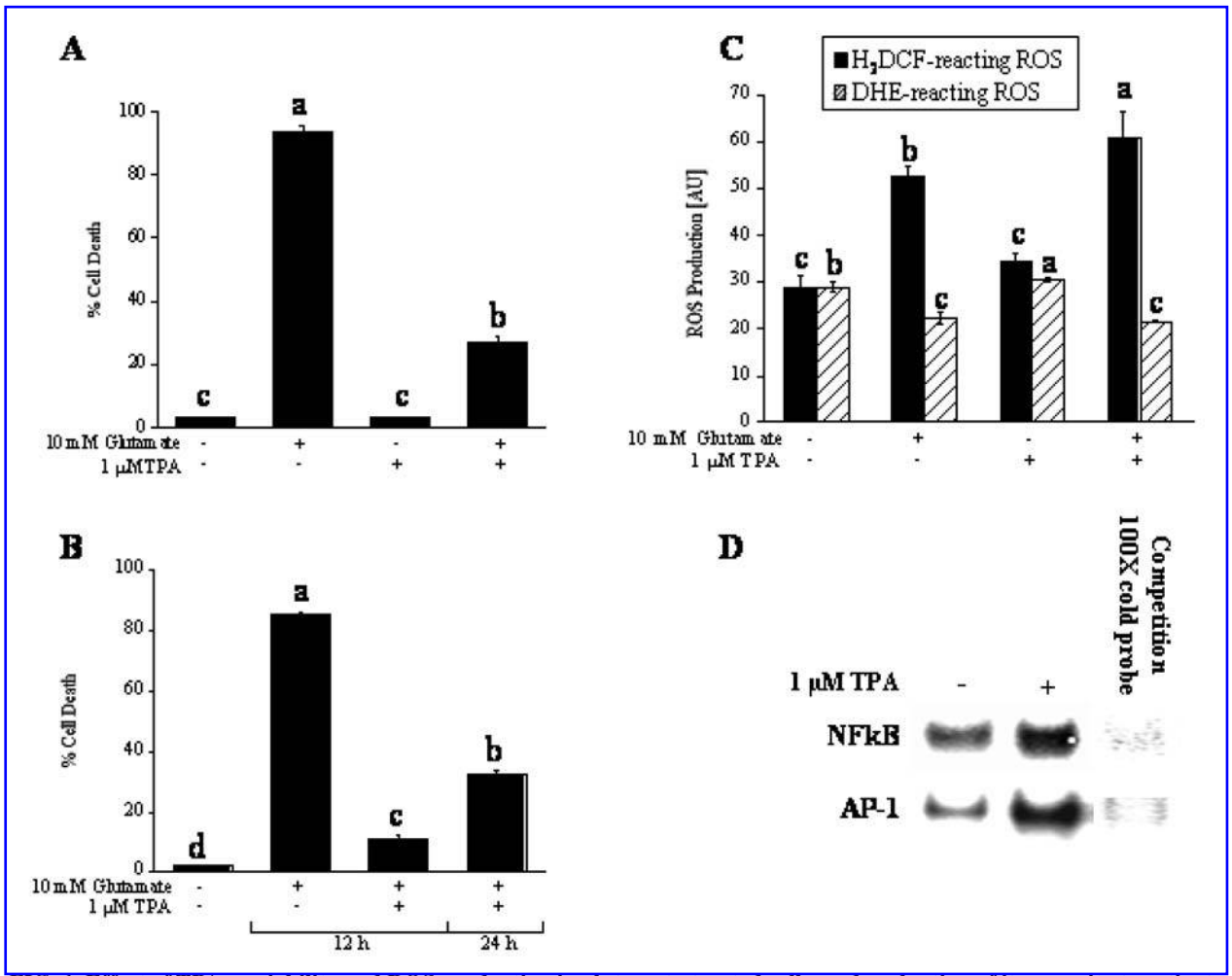

FIG. 1. Effect of TPA on viability and ROS production in glutamate-treated cells, and evaluation of its capacity to activate NFκB and AP-1. **(A)** Protective effect of TPA when added to the culture medium 5 min before a 12-h glutamate treatment. Means with different letters differ at $p < 0.05$. **(B)** TPA protective effect 12 and 24 h after glutamate treatment. **(C)** Changes in H_2DCF- and DHE-reacting ROS in the cells after 6-h treatment with TPA and glutamate. Means with different letters in each group differ at $p < 0.05$. **(D)** Upregulation of NFκB and AP-1 DNA-binding activity 6 h after TPA treatment is shown by a representative EMSA gel run of three independent experiments. Competition with a cold probe was performed to show specificity.

cell survival. TPA itself induced an increase in NFκB and AP-1 nuclear activity 6 h after treatment, as measured by EMSA (Fig. 1D).

Role of NFκB in the protective effect of TPA against glutamate's neurotoxic effects

Treatment of the cells with glutamate for 6 h did not facilitate changes in NFκB nuclear activity (Fig. 2A, top). The effect of glutamate and TPA together on NFκB nuclear activity was not significantly different from that of TPA alone (Fig. 2A, bottom).

To suppress NFκB nuclear activity, we transfected the cells with the vector IκBαΔN, which encodes a mutant IκBα. This mutation prevents phosphorylation of IκBα, its degradation, and its release from NFκB. Western blot analysis of IκBα confirmed overexpression (increased protein levels), and EMSA of NFκB showed decreased nuclear activity (Fig. 2B, top). A decreased level of NFκB nuclear activity also was observed in cells transfected with IκBαΔN and treated with glutamate with or without TPA for 6 h, indicating that glutamate and TPA were unable to facilitate NFκB activation in the transfected cells (Fig. 2B, middle and bottom). However, suppression of NFκB activity did not change the vulnerability of HT4 cells to glutamate (Fig. 2C). To show further that NFκB does not play a protective role in glutamate-induced HT4 cell death, IκBαΔN-overexpressing cells were treated with glutamate and TPA together. Although NFκB nuclear activity was suppressed, TPA was still capable of protecting the cells from glutamate toxicity (Fig. 2D).

Role of AP-1 in the protective effect of TPA against glutamate's neurotoxic effects

Glutamate treatment for 6 h led to a significant increase in AP-1 nuclear activity (Fig. 3A, top). A synergistic increase in

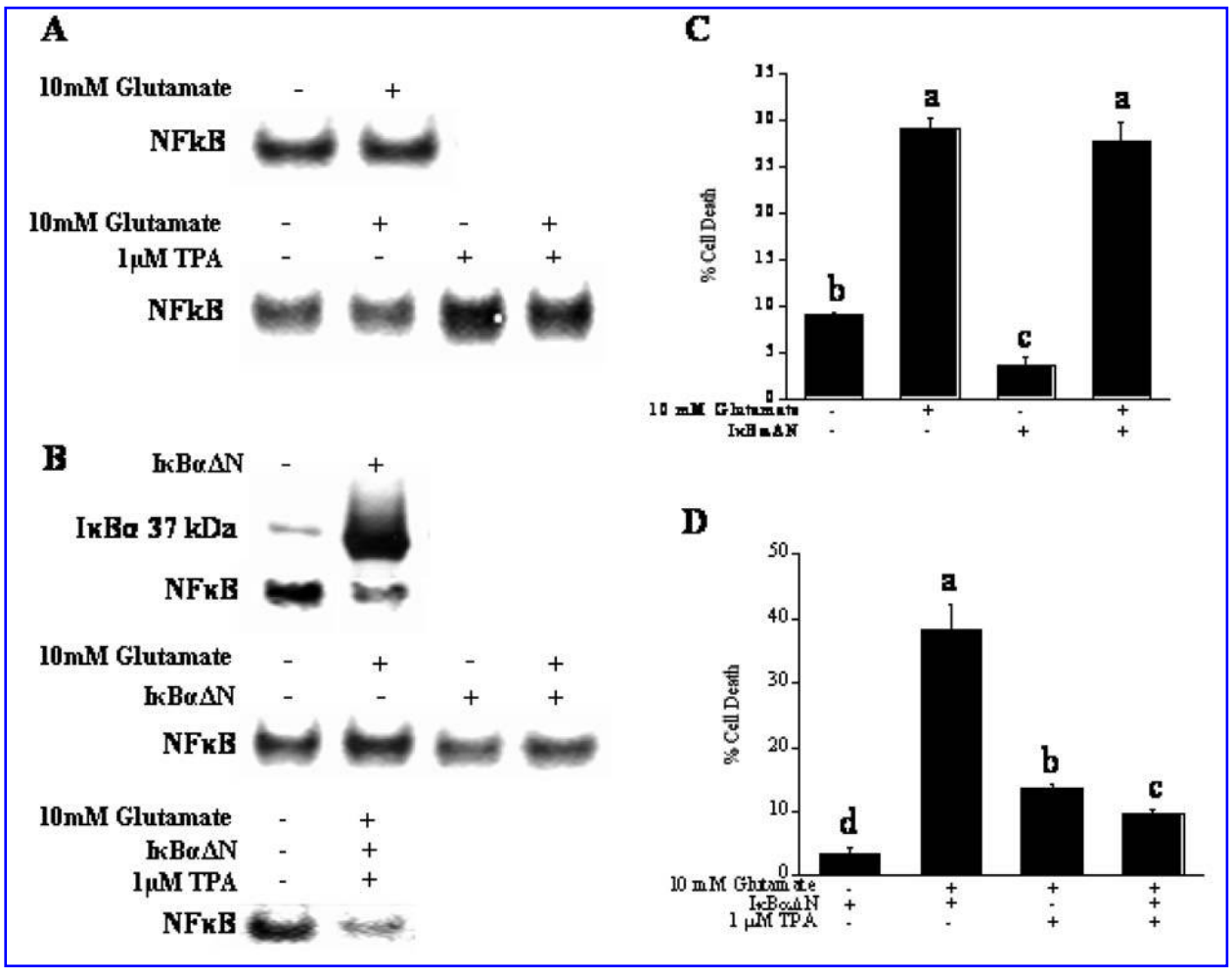

FIG. 2. Involvement of NFκB DNA nuclear activity in glutamate-induced cell death. **(A)** The effect of a 6-h treatment with glutamate alone (top) or together with TPA (bottom) on NFκB DNA-binding activity, as shown by a representative EMSA gel run of three independent experiments. **(B)** Top: Cells were transiently transfected with an empty vector (control) or IκBαΔN as described in Materials and Methods. Suppression of NFκB was verified by western blot analysis of IκBα and EMSA analysis of NFκB DNA-binding activity. Middle: EMSA analysis of NFκB DNA-binding activity performed in trasfected cells treated with glutamate. Bottom: EMSA analysis of NFκB in 6 h glutamate treated cells in the presence of TPA. **(C)** Loss of cell viability evaluated in transfected cells treated with glutamate for 12 h. Means with different letters differ at $p < 0.05$. **(D)** Loss of cell viability was measured 12 h after treatment with glutamate, TPA or both. Means with different letters differ at $p < 0.05$.

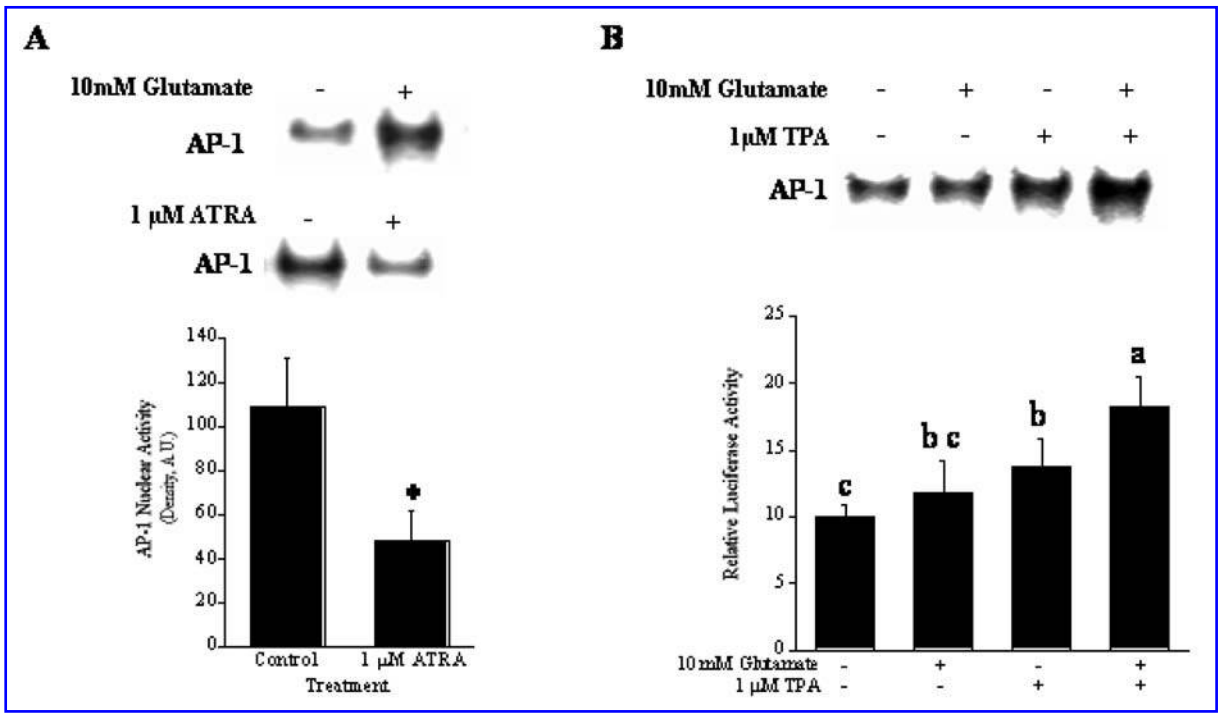

FIG. 3. AP-1 DNA nuclear activity is upregulated by glutamate and TPA and downregulated by ATRA. **(A)** AP-1 DNA-binding activity was measured by EMSA as is shown by a representative gel run of three independent experiments. Top: Glutamate treatment for 6 h induces an increase in AP-1 nuclear activity, relative to the control. Bottom: Nuclear activity of AP-1 measured in cells treated with ATRA for 24 h is shown by a representative EMSA gel run. The bar graph shows that the inhibitory effect of ATRA was significant. $*p < 0.05$, significantly different compared with control. **(B)** Treatment with TPA and glutamate for 6 h induces a synergistic increase in AP-1 DNA-binding activity in the cells. Top: EMSA analysis. Bottom: AP-1 reporter assay. Relative luciferase activity was determined by the ratio of luciferase to β-Gal. Means with different letters differ at $p < 0.05$.

AP-1 activity was observed after cells were treated with glutamate in the presence of TPA (Fig. 3B, top). To directly tie the observation of increased AP-1–binding activity with transcriptional activity, a reporter assay was performed (Fig. 3B, bottom). The increase in AP-1 activity was correlated with higher H_2DCF-reacting ROS production (Fig. 1C).

To suppress AP-1 nuclear activity, we treated the cells with ATRA. ATRA leads to activation of RARs, which have been shown to heterodimerize with components of AP-1 to inhibit its transcription-regulatory activity (6, 39, 49). AP-1 inhibition by ATRA was confirmed by EMSA (Fig. 3A, bottom, representative picture and graph). ATRA added 24 h before glutamate decreased AP-1 activity relative to cells treated with glutamate alone (Fig. 4A, top).

To study the effect of inhibited AP-1 nuclear activity on cellular redox status, H_2DCF-reacting ROS levels were measured. ATRA itself did not change ROS production compared with control, untreated cells. However, in glutamate-mediated ROS elevation, ATRA significantly attenuated ROS production (Fig. 4A, bottom). Conversely, levels of DHE-reacting ROS increased in the presence of ATRA. Under glutamate exposure, superoxide is converted to hydrogen peroxide to activate the AP-1 pathway, whereas ATRA somehow interferes with this process. Decreased H_2DCF-reacting ROS levels would be expected to have a protective effect on cells. However, increased susceptibility of HT4 cells to glutamate toxicity was observed (Fig. 4B). These data indicate a protective role of AP-1 in glutamate cytotoxicity. To further show that the effect is specific to the AP-1 pathway, we used a decoy oligonucleotide assay. Cells transfected with the AP-1 consensus sequence were more sensitive to glutamate-induced cell death than were cells transfected with scrambled DNA (Fig. 4C).

The inhibitory effect of ATRA on AP-1 activity is thought to be through increased RARα activity, and not via a direct effect of ATRA itself. Indeed, overexpression of RARα sensitized the cells to glutamate cytotoxicity (Fig. 5A), indicating direct involvement of the AP-1 pathway in attenuating the toxic effect of glutamate. Such overexpression also suppressed glutamate-induced H_2DCF-reacting ROS production (Fig. 5B). A direct correlation was found between peroxides levels in the cells, AP-1 nuclear activity, and cell viability.

Further to examine the implications of AP-1 activation in glutamate-induced HT4 cell death, we studied the effect of its inhibition on the viability of cells treated with glutamate and TPA together. Consistent with the ATRA- and RARα-mediated increase in the cells' vulnerability to glutamate, the protective effect of TPA was diminished when AP-1 nuclear activity was inhibited (treatment of TPA and glutamate in the presence of ATRA or RARα overexpression; Fig. 6A and B). The reduced AP-1 nuclear activity and the decline in the protective effect of TPA were accompanied by a decrease in H_2DCF-reacting ROS levels (Fig. 6C), indicating a link between these cellular events.

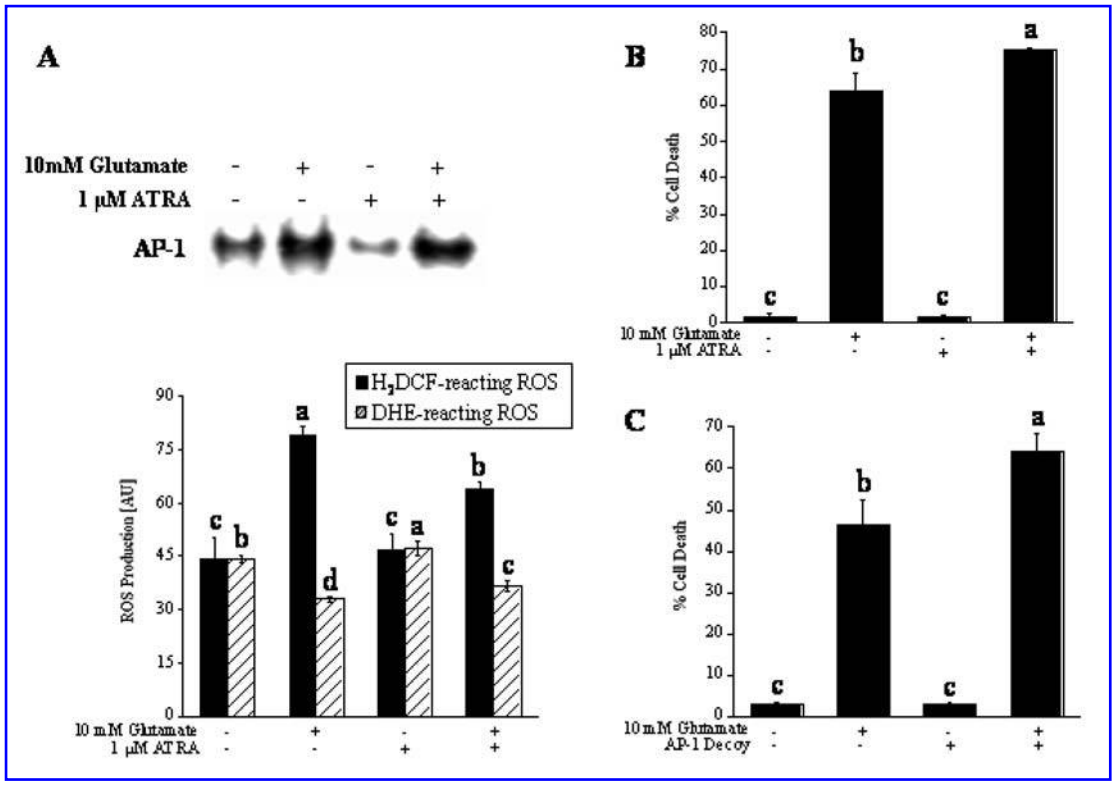

FIG. 4. Inhibition of AP-1 nuclear activity decreases ROS production and increases cell vulnerability to glutamate. Glutamate was added to the cells 24 h after ATRA treatment. **(A)** Top: AP-1 DNA-binding activity was measured 6 h after glutamate treatment, as shown by a representative EMSA gel run of three independent experiments. Bottom: Production of H_2DCF- and DHE-reacting ROS in the cells evaluated 6 h after glutamate treatment. Means with different letters in each group differ at $p < 0.05$. **(B)** Loss of cell viability was measured 10 h after glutamate treatment. Means with different letters differ at $p < 0.05$. **(C)** AP-1 nuclear activity was inhibited by decoy-oligonucleotide as described in Materials and Methods. Loss of cell viability was measured 15 h after glutamate treatment. Means with different letters differ at $p < 0.05$.

DISCUSSION

Increased production of ROS plays an important role in glutamate-induced excitotoxicity-independent cell death. This is supported by many studies that have shown protective effects of various antioxidants, such as vitamin E (47) and selenium (34). Tocotrienols have been found to be highly potent inhibitors of the death pathway because of direct interaction with components of the involved signal-transduction pathway [*e.g.*, c-Src and 12-Lox (15, 40)]. It is now well established that ROS act as second messengers in this death process. NFκB and AP-1 are suggested to be downstream signals that are redox regulated (41, 44). According to this study, the role of the transcription factor AP-1, but not NFκB, is probably to support survival in this model.

Here we showed that glutamate increases H_2DCF-reacting ROS production but does not lead to activation of NFκB in HT4 cells beyond baseline activity. Studies focusing on glutamate-mediated receptor toxicity have reported activation of this transcription factor (12, 37). However, it was found that NFκB is constitutively expressed in neurons (7, 13), and even basal levels of glutamatergic synaptic activity are sufficient to activate NFκB (23, 25). Therefore, we hypothesized that in HT4 cells, the basal expression of NFκB is already high and cannot be further activated. However, TPA increased NFκB nuclear activity. Nevertheless, the additive effect of TPA and glutamate (*i.e.*, increased ROS production) did not result in increased NFκB activation relative to TPA alone. Thus, the results indicate that the pathway of NFκB activation in HT4 cells is probably not mediated by an increase in ROS levels. Several researchers have reported that H_2O_2-induced NFκB activation is highly dependent on cell type, whereas others have suggested that endogenous cellular ROS production cannot serve as a second messenger for NFκB activation (8).

We showed that in contrast to NFκB, glutamate-mediated oxidative stress is accompanied by activation of AP-1. Many studies have shown that ROS upregulates AP-1 activity (33, 35, 53). AP-1 activation not only responds to ROS levels, but also can also modulate their production by activating the transcription of enzymes that generate various kinds of ROS [*e.g.*, manganese superoxide dismutase (MnSOD) and nitric oxide synthase (20, 32)]. We showed that under glutamate treatment, superoxide is probably converted to hydrogen peroxide to activate the AP-1 pathway. Adding TPA and glutamate together to the cells led to an elevation in H_2DCF-reacting ROS production, which was accompanied by a strong increase in AP-1 nuclear activity. In addition, inhibition of AP-1 activity by ATRA resulted in reduced levels of H_2DCF-reacting ROS. Thus, the interplay between ROS production and AP-1 activity may be driven by a positive feedback loop

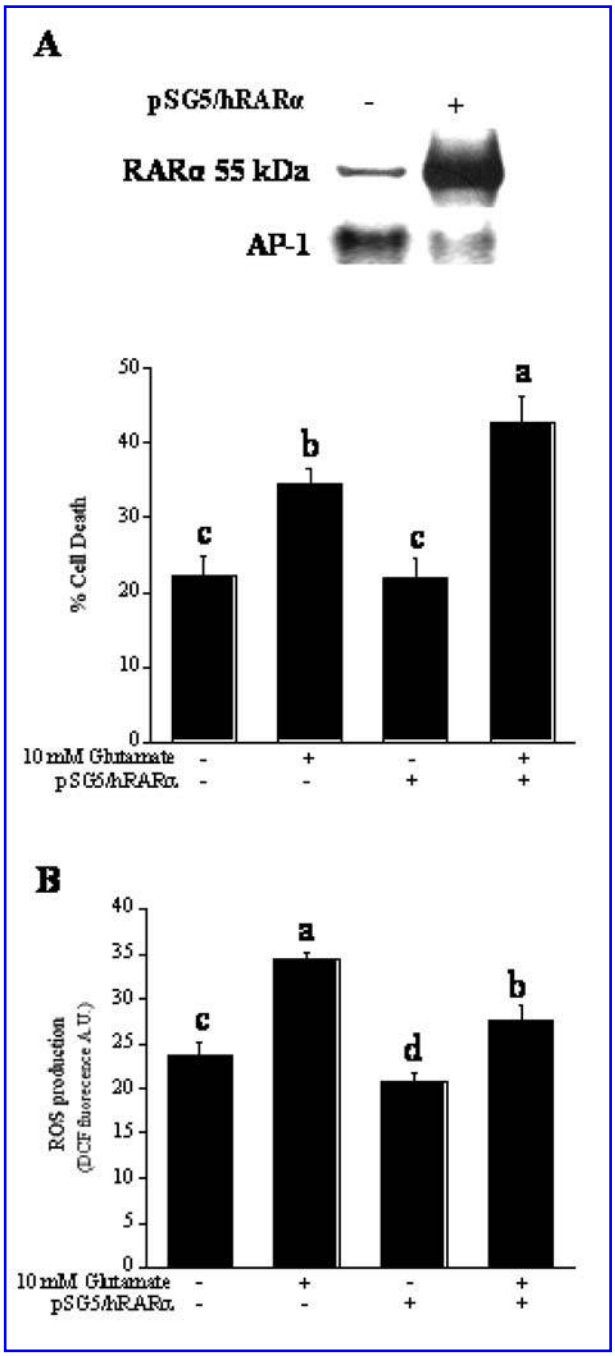

FIG. 5. Overexpression of RARα decreases ROS production and sensitizes cells to glutamate toxicity. Cells were transiently transfected with an empty vector (control) or with human RARα, as described in Materials and Methods. **(A)** Top: The transfection was verified by Western blot analysis of RARα and EMSA for AP-1 nuclear activity. Bottom: At 24 h after transfection, glutamate was added for 15 h and loss of cell viability was measured. Data are representative of three independent experiments. Means with different letters differ at $p < 0.05$. **(B)** At 24 h after transfection, cells were treated with glutamate for 6 h and the production of H_2DCF-reacting ROS in cells was evaluated. Means with different letters differ at $p < 0.05$.

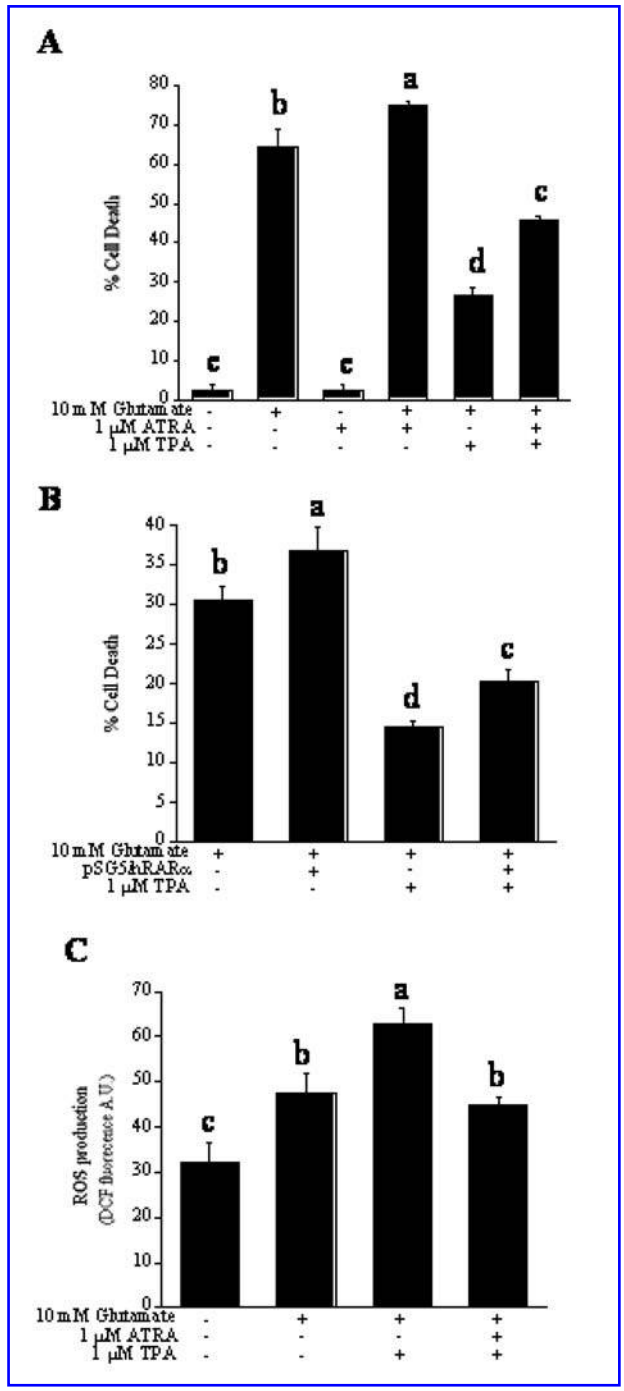

FIG. 6. Inhibition of AP-1 nuclear activity abrogates the protective effect of TPA against glutamate toxicity and attenuates cellular ROS production. **(A)** Loss of cell viability evaluated 10 h after glutamate treatment in cells treated with ATRA and TPA, as indicated in the figure. Means with different letters differ at $p < 0.05$. **(B)** Cells were transiently transfected with an empty vector (control) or with human RARα. The loss of cell viability was evaluated 15 h after treatment with glutamate and TPA, as indicated. Means with different letters differ at $p < 0.05$. **(C)** Cells were treated with ATRA, TPA and glutamate, as indicated in the figure. Production of H_2DCF-reacting ROS in the cells measured 6 h after glutamate treatment. Means with different letters differ at $p < 0.05$.

of increasing peroxides levels leading to further activation of the AP-1 pathway, which, in turn, leads to the synthesis of AP-1–responsive genes. The expression of these genes may further convert superoxide to hydrogen peroxide, in an attempt to protect the cells against glutamate. Indeed, overexpression of MnSOD partially protected the cells against glutamate treatment (data not shown).

Our current understanding of the implications of NFκB and AP-1 activation in glutamate cytotoxicity is controversial. A protective role of NFκB has been found in ischemic brain injury (36), in seizures and excitotoxic glutamate in the hippocampus (52), and in neurodegenerative disorders such as Alzheimer and Parkinson diseases (24). Our study showed that inhibition of NFκB by a specific suppressor did not result in increased cell vulnerability to glutamate. Moreover, TPA protected the cells, even when NFκB activity was suppressed. These results indicate that NFκB has little or no protective role in this model of glutamate-induced receptor-independent cell death.

Initially, we thought that increased AP-1 nuclear activity in glutamate cytotoxicity promotes cell death, because cycloheximide, a protein-synthesis inhibitor, protected the cells from death (2, 47, 55). Cycloheximide may prevent *c-fos* expression and AP-1 activation. However, it could be that cycloheximide prevents the expression of proteins that are involved in the death process and that are probably expressed independently of AP-1. Our study demonstrates a protective role for AP-1. Inhibition of AP-1 activity resulted in increased susceptibility of the cells to glutamate and decreased the ability of TPA to protect them. Consistent with this is the study of Rogers *et al*. (33) showing that *c-fos* expression in rat cortical neurons is protective. However, the increase in AP-1 nuclear activity after glutamate treatment is not sufficient to prevent cell death.

Our results demonstrate that ROS, regardless of their deleterious effects, can activate a protective signaling pathway through activation of AP-1. This protective effect is probably activated by H_2O_2. We also show that NFκB does not play a role in glutamate-mediated excitotoxicity-independent cell death. Further studies are required to develop a better understanding of the signaling cascade that leads to AP-1 activation and the interaction between AP-1 and ROS production in this model.

Future perspective

TPA, a PKC activator, protects neuronal cells from death induced by oxidative stress (45). Maher *et al*. (21) found that TPA differentially modulates the levels of different members of the PKC family. A specific role of PKCδ in the death process was suggested. Downstream effects of TPA involve the rapid activation of extracellular-signal-regulated kinases (ERKs), which in turn results in both inactivation of p38 mitogen–activated protein kinase (MAPK) and activation of *c-jun* NH_2 terminal kinase (JNK) (17, 21). Glutamate facilitates the expression of one or more proteins that promote cell death, as cycloheximide protects the cells. As many of the substrates of p38 MAPK are transcription factors, it has been suggested that TPA-mediated inactivation of p38 MAPK prevents the expression of the "death-promoting proteins" (45). The fact that TPA is protective only if added in the first 4 h after glutamate treatment supports this hypothesis (5). However, the protective effect could also be due to the rapid activation of ERK and JNK, followed by downstream signals such as the expression of *c-fos* and the consequential activation of AP-1. Yet inhibition of AP-1 activity did not completely abolish the protective effects of TPA, indicating the involvement of additional factors in this process. Studies designed to characterize the signals that promote the protective effect of TPA may also contribute to our understanding of glutamate toxicity.

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ABBREVIATIONS

AP-1, activating protein 1; ATRA, all-*trans*-retinoic acid; DCF, dichlorofluorescein; DHE, dihydroethidium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EMSA, electromobility-shift assay; GSH, glutathione; IκBα, inhibitory κappa B α; ONPG, *O*-nitrophenyl-β-*d*-galactopyranoside; NFκB, nuclear factor kappa B; PBS, phosphate-buffered saline; PI, propidium iodide; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; RARα, retinoic acid receptor α; ROS, reactive oxygen species; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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