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CELLULAR AND MITOCHONDRIAL CHANGES IN GLUTAMATE-INDUCED HT4 NEURONAL CELL DEATH

O. TIROSH,*† C. K. SEN,†‡§ S. ROY‡ and L. PACKER*

*Molecular and Cell Biology, and ‡Lawrence Berkeley National Laboratory, University of California, Berkeley, CA 94720-3200, U.S.A.

Abstract—Elevated levels of extracellular glutamate are neurotoxic. The cytotoxic property of extracellular glutamate is known to mediate two primary mechanisms, excitotoxicity and excitotoxicity-independent processes. The excitotoxicity-independent pathway was investigated in the current study in a mouse hippocampal-derived HT4 cell line. Exposure of HT4 cells to glutamate for 12 h induced loss of cell viability preceded by rapid loss of intracellular reduced glutathione followed by accumulation of intracellular reactive oxygen species, elevation of intracellular Ca^{2+}, progressive loss of mitochondrial membrane potential swelling and loss of mitochondrial outer membrane integrity. Glutamate-induced loss of DNA integrity has been detected. The antioxidants α-tocopherol and trolox, mitochondrial calcium uniporter inhibitor Ruthenium Red and protein synthesis inhibitor cycloheximide all showed protection against glutamate-induced toxicity. None of the protective agents except for α-tocopherol controlled the glutamate-induced reactive oxygen species build-up. However, these cell death regulators prevented the glutamate-induced mitochondrial damage and regulated glutamate-induced increase in intracellular Ca^{2+}. Carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone, a mitochondrial uncoupler, partially protected against glutamate-induced cell death and mitochondrial damage, while the mitochondrial ribosomal inhibitor chloramphenicol and extracellular Ca^{2+} chelator ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid did not protect the cells against glutamate treatment.

The results of this study demonstrated that mitochondrial dysfunction was a key event in the excitotoxicity-independent component of neuronal cell death. Reactive oxygen species accumulation and glutathione depletion were prominent in glutamate-treated cells; however, these events were not direct mediators of cell death. © 2000 IBRO. Published by Elsevier Science Ltd.

Key words: antioxidants, redox, reactive oxygen species, protein synthesis, intracellular calcium.

Glutamate functions as a major excitatory neurotransmitter in the brain. Elevated levels of extracellular glutamate, however, are neurotoxic.[10] The cytotoxic property of extracellular glutamate is known to be mediated by two primary mechanisms, excitotoxicity and excitotoxicity-independent processes.[8,10,46] Oxidative stress is thought to play a role in the excitotoxicity-independent pathway of glutamate-induced cell death.[46] Several studies have attempted to characterize the excitotoxicity-independent events involved in glutamate-induced death.[14,19,27–29,31,32,41] A variety of *in vitro* models (e.g., HT4, HT22, PC12 and C6 cells), lacking a fully functional excitoxicity pathway, have been used to specifically study excitotoxicity-independent glutamate-induced cell death.[14,17,27–29,32,34,46] These studies have consistently shown that glutamate-induced death is preceded by loss of intracellular glutathione and accumulation of intracellular reactive oxygen species (ROS). In addition, it has been observed that a wide variety of structurally unrelated antioxidants prevent glutamate-induced death of these cells.[17,31,34] These data have led to the hypothesis that oxidative stress plays a central role in glutamate-induced excitotoxicity-independent cell death. However, cycloheximide, a protein synthesis inhibitor which inhibits the toxic effect of glutamate, does not block the decrease in cellular glutathione content; therefore, the exact nature of the death mechanisms remains largely unknown.[14]

Mitochondria play a central role in several pathways leading to neuronal cell death.[9,20,21] Apoptosis is associated with mitochondrial permeability transition (MPT) pore opening, loss of mitochondrial membrane potential (MMP), and release of apoptogenic factors such as cytochrome *c* to the cytosol.[6,7,22,23,30] In a study of HT22 neuronal cells investigating the excitotoxicity-independent path of glutamate-induced cell death, a central role of mitochondria has been reported.[46] However, glutamate's effect on the MMP and the organelle structural integrity was not addressed. Although results obtained in our laboratory[17,49] as well as other laboratories[28,46] clearly show that glutamate-induced death of HT cells is preceded by elevation of intracellular ROS, there is no straightforward evidence establishing a causative role of ROS in the death pathway. In addition to that, the temporal loss of the MMP and the mitochondrial structural changes underlying such mitochondrial dysfunction, had not yet been described in excitoxicity-independent glutamate-induced cytotoxicity. The objective of the current study was to develop an understanding of the intracellular events involved in glutamate-induced death of HT4 cells with emphasis on elucidating the putative role of mitochondria and ROS.

EXPERIMENTAL PROCEDURES

Materials

Sources were as follows. L-Glutamic acid monosodium salt, cycloheximide, chloramphenicol, Ruthenium Red, carbonyl cyanide

†O. Tirosh and C. K. Sen contributed equally to this work and are joint first authors.

§To whom correspondence should be addressed. Tel.: +1-510-4866758; fax: +1-510-6442341.

E-mail address: cksen@socrates.berkeley.edu (C. K. Sen).

Abbreviations: AM, acetomethoxyl; $[Ca^{2+}]_i$, intracellular free calcium ions; CsA, cyclosporin A; DCF, dichlorofluorescein; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone; GSH, reduced glutathione; H_2DCF-DA, dichlorodihydrofluorescein diacetate; HPLC, high-performance liquid chromatography; JC-1, 5,5′,6,6′-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide; MMP, mitochondrial membrane potential; MPT, mitochondrial permeability transition; PBS, phosphate-buffered saline; ROS, reactive oxygen species.

p-trifluoromethoxyphenyl-hydrazone (FCCP), cytochrome *c*, potassium cyanide, sodium succinate, EGTA, cylosporin A (CsA) (Sigma, St Louis, MO, U.S.A.); *d*-α-tocopherol 98% (Henkel Corporation, La Grange, IL, U.S.A.); trolox (Aldrich, Milwaukee, WI, U.S.A.), high glucose containing Dulbecco's modified Eagles medium (Gibco, Gaithersburg, MD, U.S.A.); fetal calf serum, penicillin and streptomycin (University of California, San Francisco, CA, U.S.A.); tissue culture dishes $100 \times 15\ mm^2$ (Becton Dickinson, Palo Alto, CA, U.S.A.); and JC-1 dichlorodihydrofluorescein diacetate (H_2DCF–DA), propidium iodide, calcium green-1, AM (Molecular Probes, Eugene, OR, U.S.A.). Material used for electron microscopy and related sample preparation was from Ted Pella, Redding, CA, U.S.A.).

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, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. Semi-confluent cells were trypsinized and subcultivated 5 ml per culture dish at a concentration of 6.8×10^4 cells per ml. Following 24 h of seeding, the culture medium was replaced with fresh medium supplemented with serum and antibiotic as stated above. The cells were then exposed to 10 mM glutamate according to a model of glutamate induced cytotoxicity that has been previously described for PC12 cells and HT22 cells.[41,46]

Cell viability

Cell viability was determined on the basis of lactate dehydrogenase (LDH) leakage as described previously[17,32] according to the formula of viability = LDH activity of cells in monolayer/total LDH activity (LDH activity of cells in monolayer + LDH activity of detached cells + LDH activity in the cell culture medium).

DNA integrity

HT4 cells were exposed to 10 mM glutamate for 12 h. Following the treatment, both detached and cells in a monolayer were collected and centrifuged (600 *g*, 5 min). The pellet was resuspended in a solution containing 50 μg/ml propidium iodide, 0.1% (w/v) sodium citrate and 0.1% (v/v) Triton X-100. The permeabilized cells were kept in the dark for 6 h at 4°C and their DNA integrity was analysed using a flow cytometer (XL, Coulter, FL). An argon-ion laser was used for excitation at 488 nm and the emission was recorded at 575 nm.[44,45,48] Data were collected from at least 10,000 cells.

Intracellular reactive oxygen species

Intracellular ROS were detected using H_2DCF–DA as described in our earlier work.[17] Following different treatments, the cells were washed three times with phosphate-buffered saline (PBS). Cells were detached from the monolayer using trypsin, and centrifuged (600 *g*, 5 min). The cells were resuspended in PBS and incubated with H_2DCF–DA (25 μM) for 30 min at 37°C. For the detection of intracellular fluorescence the fluorochrome-loaded cells were excited using a 488-nm argon-ion laser in a flow cytometer (XL, Coulter, FL, U.S.A.). The dichlorofluorescein (DCF) emission was recorded at 530 nm. Data were collected from at least 10,000 cells.

Determination of intracellular free calcium

Intracellular free calcium ions $[Ca^{2+}]i$ was detected using acetomethoxyl (AM) ester of calcium green-1. Following different treatments, cells were washed three times with PBS. Cells were detached from monolayer using trypsin, and centrifuged (600 *g*, 5 min). After another wash, the cells were resuspended in PBS and loaded with calcium green-1, AM (1 μM) for 20 min at room temperature. For the detection of intracellular fluorescence the fluorochrome-loaded cells were excited using a 488-nm argon-ion laser in a flow cytometer (XL, Coulter, FL, U.S.A.). The emission was recorded at 530 nm. Data were collected from at least 10,000 cells.

Intracellular glutathione

HT4 cells were washed with ice-cold PBS, treated with 2% (w/v) monocholoroacetic acid and scraped. All samples were immediately frozen in liquid nitrogen and stored at −80°C, until analysis by high-performance liquid chromatography (HPLC). Immediately before the assay, samples were thawed, vortexed and then centrifuged at 15,000 *g* for 2 min. The clear supernatant was removed and injected into the HPLC system. HPLC-EC detection of intracellular reduced glutathione (GSH) was performed using an ESA (Chelmsford, MA, U.S.A.) coulometric detector.[17]

Assessment of mitochondrial changes

Flow cytometry. Determination of mitochondrial membrane potential was carried out using the ratiometric dye JC-1. JC-1: a dual-emission potential-sensitive probe. The green fluorescent (emission at 529 nm) JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) exists as a monomer at low concentrations or at low membrane potential. However, at higher concentrations (aqueous solutions above 0.1 μM) JC-1 forms red fluorescent "J-aggregates" that exhibit a broad excitation spectrum and an emission maximum at ~590 nm. Both components of the dye are known to be sensitive to the mitochondrial membrane potential (MMP) and changes in the ratio between green to red fluorescence can provide information regarding the MMP.[12] To stain cells with JC-1, 1×10^6 cells were resuspended in 0.5 ml of medium containing 10% fetal calf serum and incubated with 10 μg/ml of JC-1 for 15 min at room temperature. The cell were then washed twice with ice-cold PBS and analysed by flow cytometry.[12]

Transmission electron microscopy. Cells in monolayer were washed with PBS and harvested by trypsinization. The cells were fixed for 6 h in a 2% v/v solution of glutaraldehyde in 0.1 M sodium cacodylate buffer (pH. 7.2) and post-fixed in 1% osmium tetroxide in sodium cacodylate buffer for 1 h. The samples were dehydrated in a series of ethanol embedded in epon-araldite. Sections (60 nm) were cut using MT 6000XL (RMC, Tucson, AZ, U.S.A.) and post-stained in 2% w/v aqueous uranyl acetate and lead tartrate. The grids were examined under a transmission electron microscope (100 SX; JEOL, Tokyo, Japan) operating at 80 kV.

Mitochondrial outer membrane integrity assay. High-amplitude mitochondrial swelling was suggested as a mechanism to disrupt the outer mitochondrial membrane and to cause release of mitochondrial pro-apoptogenic factors located in the mitochondrial intermembrane space.[35] The accessibility of cytochrome *c* to the inner mitochondrial membrane was studied by modification of the method described by Vander Heiden *et al.*[50] In brief, succinate-dependent cytochrome *c* reduction was measured. Cells were treated with glutamate. Following an appropriate time interval of treatment, cells in monolayer were washed twice with PBS, gently scraped by a cell-lifter and centrifuged (600 *g*, 5 min). The cells were resuspended in hypotonic 50 mM phosphate buffer (pH 7.5) containing 0.2 mM of potassium cyanide to inhibit cytochrome oxidase activity. Reduction of cytochrome *c* in the presence of succinate as substrate for mitochondrial respiration was followed spectrophotometrically at 550 nm.[15] The final concentrations of cytochrome *c* and succinate in the reaction mixture were 0.5 mg/ml and 20 mM, respectively. In the absence of exogenous succinate, reduction of cytochrome *c* in the assay system was negligible.

Statistics

Data are reported as mean ± S.D. of at least three experiments. Comparisons between multiple groups were made by analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

Intracellular events in glutamate cytotoxicity

Exposure of HT4 cells to glutamate induced significant loss of cell viability as measured by the release of LDH from the cells to the culture medium (Fig. 1). On response to glutamate treatment, cell, started to lose viability after 6 h. Over 90% of cell death was observed following 12 h of treatment (loss of cell viability tested by LDH leakage was confirmed by propidium iodide exclusion test;[44] not shown). High basal levels of

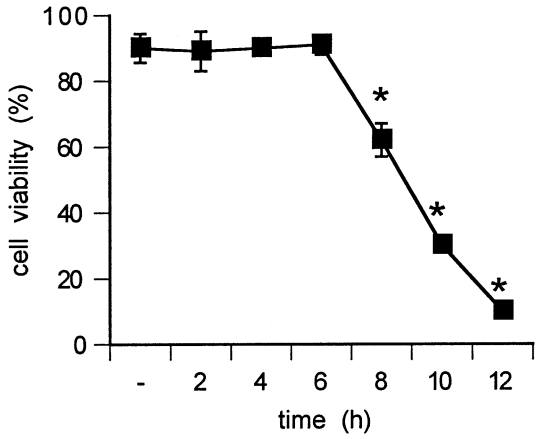

Fig. 1. Loss of cell viability following treatment with glutamate. HT4 cells were treated with 10 mM glutamate for 2–12 h and cell viability was measured by lactate dehydrogenase leakage. Control (-) represents cells cultured for 12 h with no glutamate treatment. *$P < 0.05$, lower than control cells.

GSH were detected in HT4 cells. Exposing cells to elevated level of extracellular glutamate resulted in rapid loss of intracellular GSH. Approximately 50% and 80% of intracellular GSH was depleted following 3 and 6 h of glutamate treatment, respectively (Fig. 2A). Glutathione depletion was followed by accumulation of intracellular ROS in glutamate-treated cells. Elevated level of intracellular ROS, as determined by DCF fluorescence, was detected after 3 h of glutamate treatment. The intracellular ROS level peaked after 6 h of treatment (Fig. 2B). After 8 h of glutamate treatment, however, intracellular ROS levels declined. Elevation of $[Ca^{2+}]_i$ was observed in cells challenged with glutamate. In glutamate-treated cells $[Ca^{2+}]_i$ increased progressively as a function of time (Fig. 2C).

Treatment of cells with glutamate for various durations resulted in progressive loss of MMP (Table 1) as determined by the ratiometric membrane potential-sensitive dye JC-1. Glutamate treatment for up to 8 h did not induce a complete obliteration of MMP. Treatment of cells with FCCP demonstrated a further loss of MMP. To evaluate mitochondrial damage and to determine whether mitochondrial outer membrane integrity was disrupted following exposure to high levels of glutamate, the accessibility of exogenous cytochrome *c* to the inner mitochondrial membrane was evaluated in permeabilized cells. The rate of cytochrome *c* reduction by succinate cytochrome *c* reductase of the inner mitochondria membrane was studied. Cells exposed to glutamate for various time intervals demonstrated a progressive increase in cytochrome *c* reduction rate, indicating disruption of the outer membrane. A marked increase in cytochrome *c* reduction was observed between 6 and 8 h of glutamate treatment (Fig. 3).

Transmission electron microscographs illustrate the morphological changes in the cells and mitochondria in response to glutamate treatment for 8 h. In non-challenged control cells the mitochondria had a regular shape (Fig. 4A). Glutamate treatment for 8 h resulted in substantial mitochondrial changes, which were observed before any typical apoptotic nuclear changes had appeared (Fig. 4B). Large and optically less dense mitochondria with spare cristae were noted in these cells, indicating swelling and mitochondrial degeneration. Discontinuity of the mitochondrial outer membrane is illustrated (Fig. 4C).

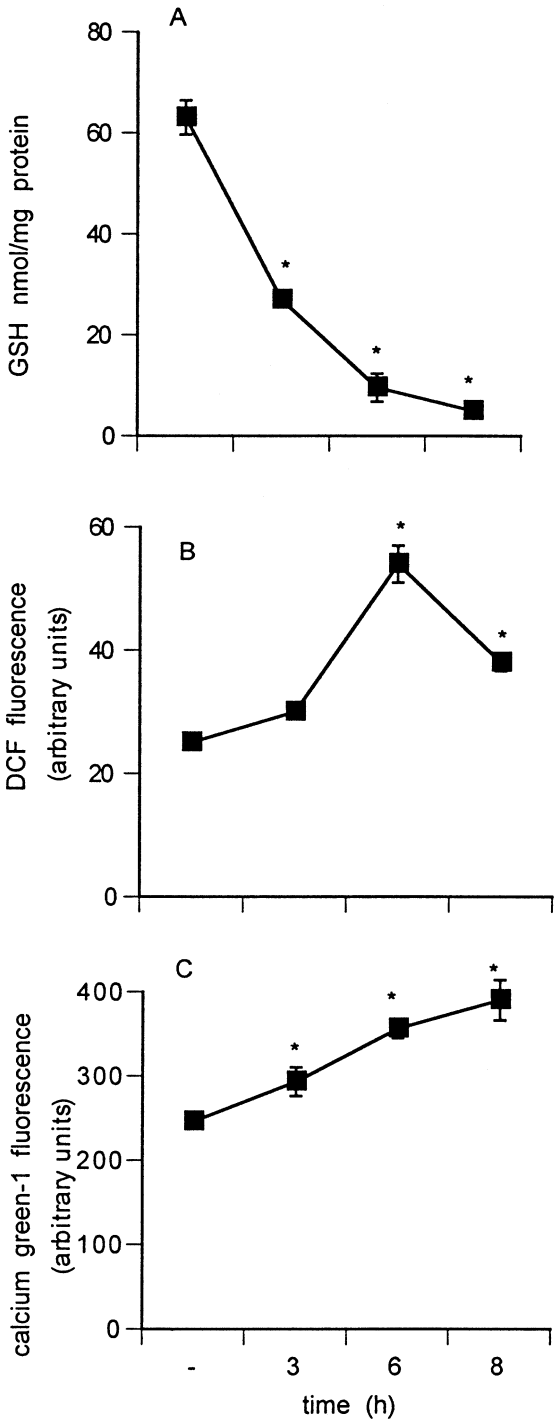

Fig. 2. Kinetic of intracellular events in HT4 cells treated with glutamate. (A) Reduced glutathione content of HT4 cells. GSH was extracted under acidic conditions and analysed by HPLC-EC. Control (-) represents cells not treated with glutamate. *$P < 0.05$, lower than control cells. (B) Intracellular ROS production. Cells treated or not with glutamate were loaded with an oxidation sensitive fluorescent probe, dichlorodihydrofluorescein diacetate (H_2DCF-DA). Fluorescence of the oxidized product DCF was analysed using a flow cytometer. *$P < 0.05$, higher than control cells. (C) Measurements of intracellular cytosolic free calcium. Cells treated or not with glutamate were loaded with the cell-permeable calcium-sensitive probe calcium green-1, AM, and were analysed by flow cytometry. *$P < 0.05$, higher than control cells.

Inhibition of the death pathway

Cell viability. The relative efficacy of selected antioxidants

Table 1. Changes in mitochondrial membrane potential

Treatment	Mitochondrial ΔΨ in HT4 cells (relative to control)
No glutamate 8 h (control)	1 ± 0.09
No glutamate 8 h + FCCP	0.22 ± 0.04*
Glutamate 3 h	0.83 ± 0.04*
Glutamate 6 h	0.52 ± 0.05*
Glutamate 8 h	0.32 ± 0.02*
Glutamate 8 h + FCCP	0.2 ± 0.01†
Glutamate 12 h (dead cells)	0.12 ± 0.002*

HT4 cells treated with glutamate were stained with the ratiometric dye JC-1 (10 μg/ml) in regular culture medium and the mitochodrial membrane potential was determined by flow cytometry using the ratio of FL2/FL1 fluorescence intensities. Open bars represent cells treated with FCCP (0.5 μM) 5 min before JC-1 staining.
*$P < 0.05$, lower than non-glutamate treated cells.
†$P < 0.05$, lower than the corresponding glutamate-treated cells.

to protect the cells from glutamate-induced death was tested. In the concentration range 1–5 μM α-tocopherol, a lipophilic antioxidant, showed remarkable protection against glutamate-induced toxicity. In contrast, the cell-permeable hydrophilic analog of α-tocopherol, trolox, failed to protect the cells against glutamate at a concentration of 5 μM. Methyl esterification of trolox conferred lipophilicity to the antioxidant. At 5 μM, methyl-trolox completely protected cells against glutamate-induced cytotoxicity (not shown). At higher concentration (50 μM) trolox partially protected HT4 cells (Fig. 5A).

The protein synthesis inhibitor cycloheximide (10 μg/ml) protected neuronal cells against glutamate challenge (Fig. 5A). However, the selective mitochodrial ribosomal inhibitor chloramephenicol[3,33] did not protect cells against glutamate challenge. The MPT inhibitor CsA[23,35] also did not protect the cells against the glutamate challenge. Ruthenium Red, an inhibitor of the mitochondrial Ca^{2+} uniporter[16], completely protected against glutamate-induced cytotoxicity (Fig. 5B). To evaluate whether extracellular calcium contributed to glutamate-induced cytotoxicity, EGTA (1 mM) was added to the cell culture medium before treating the cells with glutamate. EGTA treatment did not protect cells against the glutamate challenge, indicating that extracellular calcium is not involved in glutamate-induced cell death (Fig. 5B). The FCCP, a mitochondrial uncoupler, provided marginal protection against glutamate-induced death (Fig. 5B). Treatment of cells with FCCP (10 μM) for 12 h was slightly toxic and induced 10% loss of cell viability compared with FCCP non-treated control. In 15 h FCCP treatment resulted in the death of more than 50% of the cells (data not shown).

DNA damage and intracellular glutathione level: effect of α-tocopherol

Glutamate treatment for 12 h induced significant DNA fragmentation, a parameter that is characteristic of apoptosis (Table 2). α-Tocopherol prevented the loss of DNA integrity induced by 12-h exposure to elevated levels of extracellular glutamate (Table 2). However, α-tocopherol did not protect against glutamate-induced intracellular depletion of GSH (Table 2).

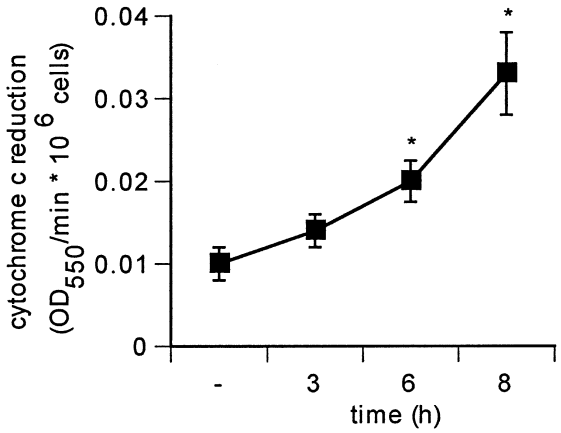

Fig. 3. Changes in mitochondrial outer membrane integrity in cells. Mitochondrial outer membrane damage as was measured by the accessibility of exogenous cytochrome *c* to the inner mitochondrial membrane and its reduction. Cells treated with glutamate were washed scraped and resuspended in hypotonic phosphate buffer. The activity of succinate cytochrome *c* reductase was evaluated spectrophotometrically at 550 nm following addition of of exogenous cytochrome *c* (0.5 mg/ml) and succinate (20 mM) *$P < 0.05$, higher than non-glutamate treated cells.

Intracellular reactive oxygen species

Significant ROS accumulation was detected in the HT4 cells treated with glutamate for 6 h (Fig. 6A, B). α-Tocopherol, a lipophilic antioxidant, and the water-soluble analog trolox were tested for the control of glutamate-induced accumulation of intracellular ROS. α-Tocopherol effectively inhibited the peroxides accumulation while trolox did not (Fig. 6A). In the presence of trolox, higher levels of ROS were detected in the glutamate-exposed cells (Fig. 6A).

The effect of cycloheximide and Ruthenium Red on intracellular ROS levels were tested. Although these compounds prevented glutamate-induced cell death they did not prevent the accumulation of ROS (Fig. 6A). Modulators of mitochondrial function, such as chloramphenicol, which is known to suppress the electron transport chain[33] or the mitochondrial uncoupler FCCP did not prevent the ROS accumulation at 6 h and high levels of intracellular ROS were detected (Fig. 6B).

Intracellular calcium. Treatment of cells with α-tocopherol prevented the glutamate-induced increase in $[Ca^{2+}]_i$ (Fig. 7A). Ruthenium Red had a moderate inhibitory effect and only partially prevented the increase in $[Ca^{2+}]_i$ (Fig. 7A). Cycloheximide also inhibited the glutamate-induced increase in intracellular $[Ca^{2+}]_i$ (Fig. 7B). Extracellular EGTA did not influence glutamate-induced changes in $[Ca^{2+}]_i$ (Fig. 7B).

Protection against glutamate-induced mitochondrial damage. Glutamate induced a loss of mitochondrial outer membrane integrity, as evaluated by the increased in cytochrome *c* reduction resulting from higher accessibility of exogenous cytochrome *c* to the inner mitochondrial membrane.

Fig. 4. Morphological changes in response to glutamate challenge. (A) Control cells with no glutamate. Mitochondria appeared with regular shape and optical density. (B) Glutamate-treated (8 h) cell. The mitochondria appeared to be swollen (larger in size with low optical density). (C) Glutamate (10 mM) treatment for 8 h resulted in swollen and degraded mitochondria as was observed with higher magnification. Mitochondrial outer membrane disruption is observed. Scale bars = 1.85 μm (A, B), 0.21 μm (C).

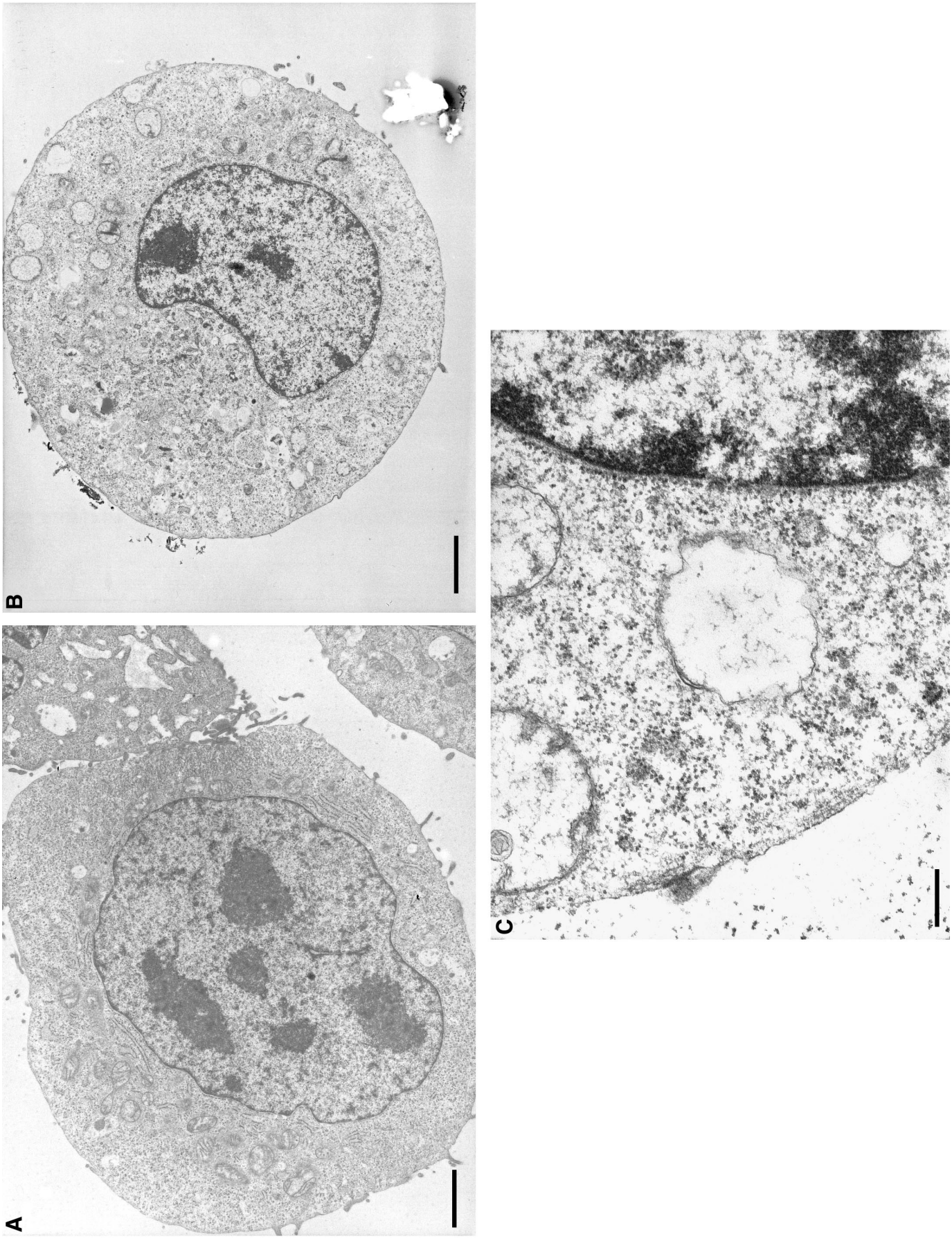

Fig. 4.

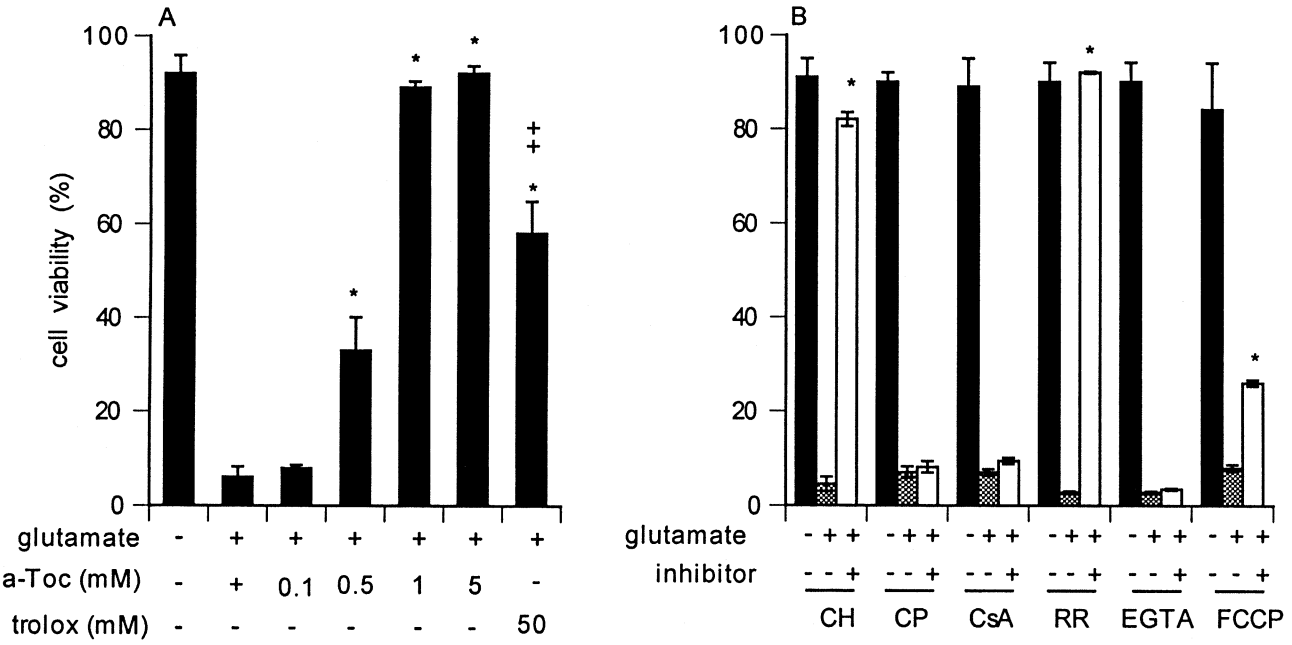

Fig. 5. Glutamate-induced loss of cell viability and its regulation. (A) Inhibition of glutamate toxicity (treatment for 12 h) by antioxidants as evaluated by determination of LDH leakage. Effect of α-tocopherol (a-Toc) and trolox when added to the cell culture medium 5 min before the glutamate treatment. *$P < 0.05$, higher than cells treated with glutamate alone. ‡$P < 0.05$, lower than the glutamate-treated cells in the presence of 5 μM a-Toc. (B) Effect of various inhibitors on glutamate toxicity. The inhibitors that were used are 10 μg/ml of cycloheximide (CH), 50 μg/ml of chloramphenicol (CP), 10 μM of CsA, 20 μM of Ruthenium Red (RR), 1 mM of EGTA and 10 μM of FCCP. None of the compounds tested was toxic to cells except for FCCP (see Results). *$P < 0.05$, higher than glutamate-treated cells.

Glutamate-induced loss of outer mitochondrial membrane integrity was prevented by α-tocopherol co-treatment with glutamate. α-Tocopherol added 6 h after the glutamate treatment, however, did not prevent the damage (Fig. 8A). FCCP partially protected against glutamate-induced damage to outer mitochondrial membrane integrity (Fig. 8B). Cycloheximide and Ruthenium Red completely prevented the glutamate-induced damage to the outer mitochondrial membrane (Fig. 8C, D). Chloramephenicol, a specific mitochondrial ribosomal inhibitor, had no effect on the glutamate-induced augmented

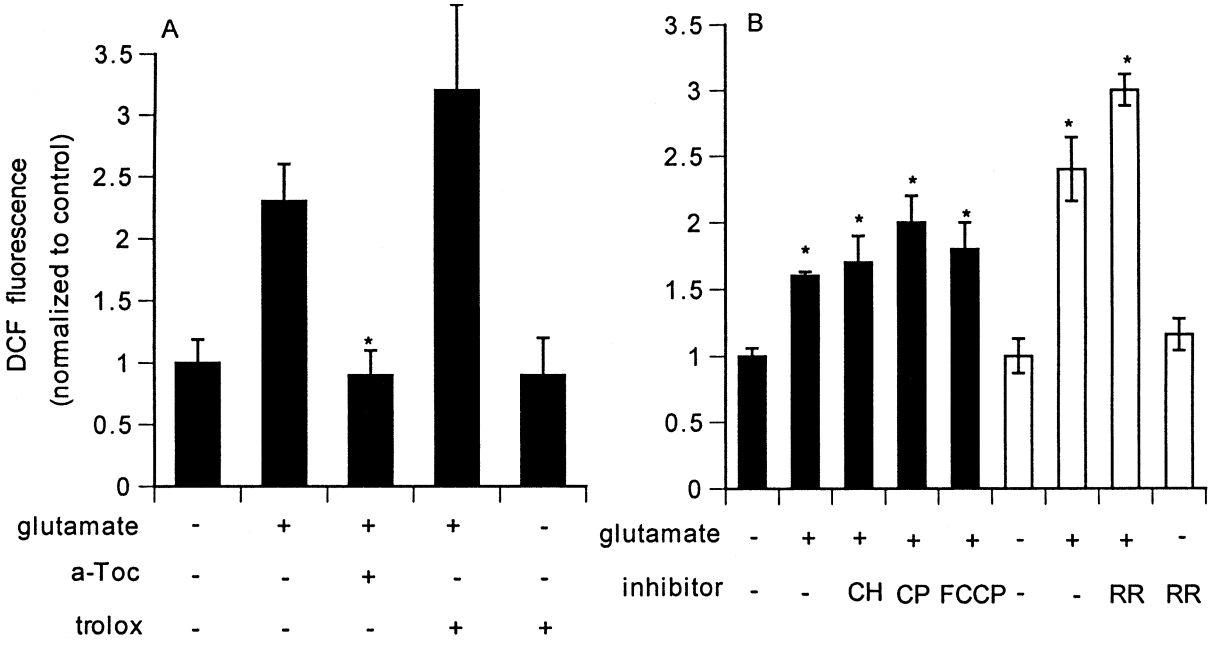

Fig. 6. Intracellular reactive oxygen species level following 6 h of glutamate treatment. (A) Effect of oxidants on intracellular ROS. DCF fluorescence was recorded using flow cytometry. *$P < 0.05$, lower than glutamate-treated cells. (B) Effect of various inhibitors on intracellular ROS. DCF fluorescence recorded from cells following exposure to glutamate for 6 h in the presence of either protein synthesis inhibitors cycloheximide (CH) or chloramphenicol (CP) used at concentrations of 10 μg/ml and 50 μg/ml, respectively, or in the presence of the mitochondrial uncoupler FCCP (10 μM) (solid bars). The effect of Ruthenium Red (RR) (20 μM) on intracellular ROS accumulation following glutamate treatment for 6 h (open bars). *$P < 0.05$, higher than non-glutamate treated controls.

Table 2. Glutamate-induced loss of DNA integrity and intracellular GSH, and effects of α-tocopherol

Treatment (A)	Fragmented DNA (percentage)
No glutamate 12 h	14.6 ± 2.6
Glutamate 12 h	47.9 ± 13*
Glutamate 12 h + α-tocopherol (5 μM)	13.99 ± 0.7†

Treatment (B)	Glutathione content (nmol/mg protein)
No glutamate 8 h	62 ± 3.4
Glutamate 6 h	9.6 ± 2.8‡
Glutamate 6 h + α-tocopherol (5 μM)	8.5 ± 0.3‡
Glutamate 8 h	5.0 ± 0.9‡
Glutamate 8 h	5.3 ± 1.5‡

Treatment (A): glutamate treatment for 12 h induced loss of DNA integrity as evaluated by the level of fragmented DNA. Cells following glutamate treatment were collected, permeabilized in hypotonic solution and stained with propidium iodide. The percentage of fragmented DNA was evaluated using flow cytometry based on the lower fluorescence intensity recorded from fragmented DNA compared with diploid-intact DNA. α-Tocopherol (5 μM) co-treated with glutamate protected against glutamate-induced DNA fragmentation.

Treatment (B): glutamate treatment for 6 h induced loss of intracellular GSH. α-Tocopherol (5 μM) co-treated with glutamate did not protect against glutamate-induced loss of GSH.

*$P < 0.05$, higher than non-glutamate-treated control.

†$P < 0.05$, lower than the glutamate-treated cell.

‡$P < 0.05$, lower compared with glutamate non-treated control.

succinate-dependent cytochrome *c* reductase activity (not shown).

Loss of mitochondrial membrane potential in relation to loss of cell viability. α-Tocopherol co-treated with glutamate significantly inhibited the loss in MMP induced by glutamate treatment for 8 h (Fig. 9A). However, α-tocopherol was ineffective when added 6 h after the glutamate treatment (Fig. 9A). Ruthenium Red co-treated or added 6 h after glutamate prevented the loss of MMP. Protection by α-tocopherol was markedly impaired when the antioxidant was added 6 h following glutamate treatment. However, Ruthenium Red provided significant protection even when added 6 or 8 h after glutamate challenge (Fig. 9B). Cycloheximide, which was observed to abrogate glutamate cytotoxicity, also prevented the glutamate-induced loss in MMP (data not shown).

DISCUSSION

Excitotoxicity and oxidative stress are two phenomena that have been described as being implicated in a wide range of disorders of the nervous system.[4] HT4 neuronal cells represent a cell line that is not sensitive to excitotoxicity. High amounts of glutamate and long-term exposure were needed to activate the death pathway in these mouse-derived hippocampal cell lines. However, the intracellular events observed in response to glutamate treatment revealed similarities between the receptor-mediated glutamate excitotoxicity and the non-receptor-mediated death. Events such as the requirement for *de novo* synthesis of proteins, disruption of $[Ca^{2+}]_i$ homeostasis, accumulation of $[Ca^{2+}]_i$ by the mitochondria, mitochondria deterioration and loss of its membrane potential described in this study are also components of the excitotoxicity-induced death pathway.[5,13,40,51,52]

Glutamate cytotoxicity

Reactive oxygen species. An elevated level of extracellular glutamate induces cell death in a unique way that has characteristics of both apoptosis and necrosis.[47] Indeed, in the HT4 model we found characteristics of both types of cell death. The apoptotic characteristics were the loss of DNA

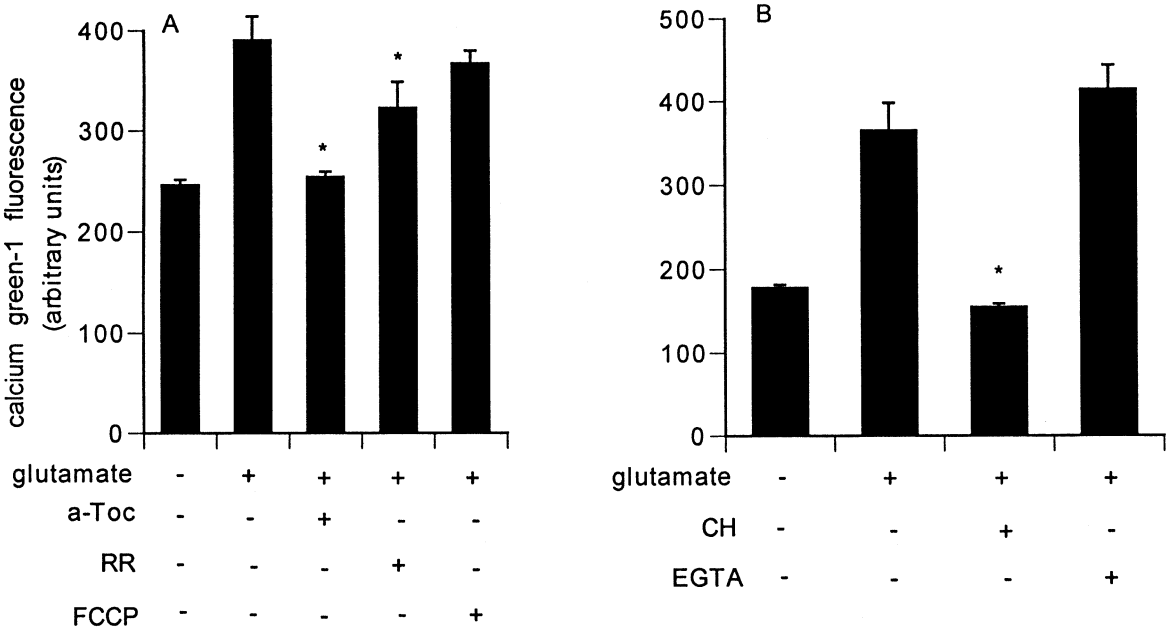

Fig. 7. Glutamate-induced changes in intacellular Ca^{2+} homeostasis. (A) Treatment of cells with glutamate for 8 h resulted in an increase in intracellular free calcium as measured in cells loaded with esterified calcium green-1. Five μM of α-tocopherol (a-Toc), 20 μM Ruthenium Red (RR) or 10 μM of FCCP was added 5 min before glutamate treatment. *$P < 0.05$, lower than glutamate-treated cells. (B) Elevation of intracellular free calcium following 8 h of glutamate treatment and the inhibitory effect of cycloheximide (CH) (10 μg/ml) or EGTA (1 mM). * $P < 0.05$, lower than glutamate-treated cells.

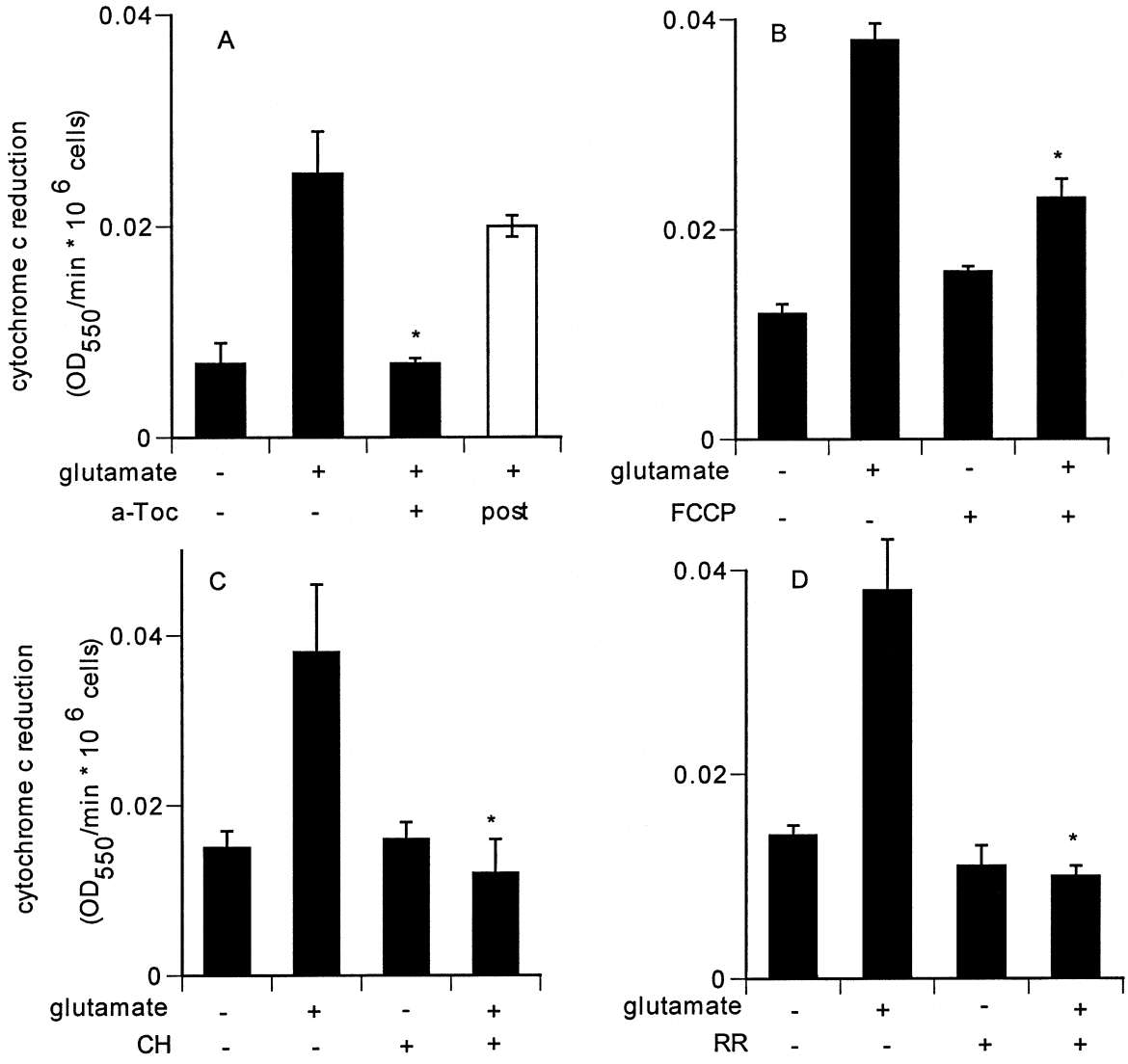

Fig. 8. Loss of mitochondrial outer membrane integrity in response to glutamate treatment Cells treated with glutamate (8 h) were washed, scraped and resuspended in hypotonic phosphate buffer. The activity of succinate cytochrome *c* reductase was evaluated spectrophotometrically at 550 nm following addition of exogenous cytochrome *c* (0.5 mg/ml) and succinate (20 mM). (A) Effect of 5 μM of α-tocopherol (a-Toc) co-treated with glutamate, Open bar represents 5 μM of a-Toc added 6 h after glutamate treatment. (B) Effect of 10 μM FCCP co-treated with glutamate. (C) Effect of 10 μg/ml cycloheximide (CH) co-treated with glutamate. (D) Effect of 20 μM of Ruthenium Red (RR) co-treated with glutamate. *$P < 0.05$, lower than glutamate-treated cells.

integrity and a requirement for protein synthesis, while loss of membrane integrity and the lack of phosphatidylserine externalization (data not shown) were the characteristics of necrosis. Genomic instability (DNA fragmentation) was studied previously in HT22 cells after exposure to 5 mM glutamate by two separate methods, agarose gel and [^{3}H]thymidine release[47]. No DNA fragmentation was observed with the first method and a minor DNA fragmentation was observed when the second method was used, leading to the conclusion that in glutamate-induced cytotoxicity there is no involvement of nuclear damage[47]. However, by using permeabilized HT4 cells stained with propidium iodide and sensitive flow-cytometric detection up to 60% of DNA fragmentation was observed in cells treated with glutamate for 12 h, indicating that the death process probably included activation of endonucleases leading to nuclear instability and DNA fragmentation.

In this current model, antioxidants were found to potently suppress the death pathway. Cell death was inhibited by the lipophilic phenolic antioxidant, α-tocopherol, more effectively than by the corresponding water-soluble analog trolox. α-Tocopherol was also effective in preventing the ROS accumulation in cells challenged with glutamate. Although the source of intracellular ROS is still elusive it is likely to originate from a lipophilic compartment in the cell. This hypothesis is supported by the observation that lipophilic antioxidants such as α-tocopherol completely prevented accumulation of intracellular ROS even under GSH-deficient conditions. Accumulation of intracellular ROS was insensitive to treatment with cycloheximide, Ruthenium Red, FCCP and trolox, all of which significantly protected the cells against glutamate-induced death. These findings indicate that accumulation of intracellular ROS and loss of cellular glutathaione do not directly contribute to the glutamate-induced cytotoxicity. However, the ability of a wide variety of structurally unrelated antioxidants to prevent glutamate-induced death to HT4 cells[49] suggests that the death pathway may include one or more redox-sensitive components.

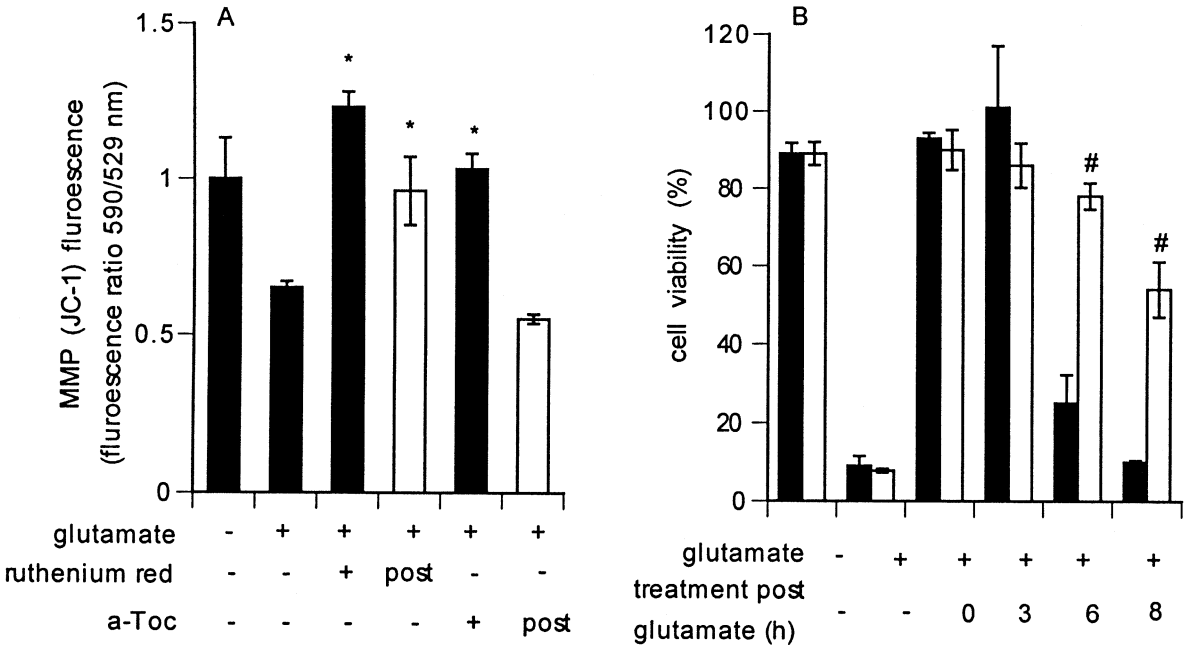

Fig. 9. Inhibition of glutamate-induced loss of mitochondrial membrane potential and cell viability. (A) HT4 cells treated with glutamate (8 h) were stained with the radiometric dye JC-1 (10 μg/ml) in regular culture medium and the MMP was evaluated by flow cytometry. The effect of 5 μM α-tocopherol (a-Toc) or 20 μM Ruthenium Red was evaluated. Solid bars represent co-treatment with glutamate. Open bars represent inhibitors added 6 h after glutamate treatment. *$P < 0.05$, higher than glutamate-treated cells. (B) Loss of viability of HT4 neuronal cells following treatment with glutamate for 12 h and the protective effect of 5 μM α-tocopherol (solid bars) or 20 μM Ruthenium Red (open bars). α-Tocopherol or ruthenium red was co-treated with the glutamate or added to the cell culture medium 3, 6 or 8 h after glutamate treatment. #$P < 0.05$, higher than the corresponding glutamate-treated cells in the presence of α-tocopherol.

Production of ROS in HT22 cells treated with glutamate was suggested to be 12-lipoxygenase dependent and a bi phasic phenomenon, and the production of ROS during the second phase was 200–400-fold above control.[28,46] However, in the HT4 cells we did not detect this second high-yield phase of ROS production and the amount of ROS produced in the glutamate-treated cells was insufficient to kill the cells, indicating that other pathways were involved in the cell death process.

Protein synthesis and calcium mobilization. De novo protein syntheses have been shown to be required in a variety of death pathways.[13,23,40] Consistently, we observed that inhibition of protein synthesis by cycloheximide protected HT4 cells against glutamate cytotoxicity. ROS, especially hydrogen peroxide, have been shown to serve as intracellular messengers.[25,26,42,43] ROS are also known to mediate calcium release from mitochondria and from other intracellular pools.[36–38] α-Tocopherol inhibited the accumulation of free calcium and ROS in the cytosol, suggesting that oxidative stress and calcium homeostasis may be strongly related. Interestingly, cycloheximide completely protected the cells against accumulation of intracellular cytosolic free calcium without any effect on the intracellular ROS build-up. This indicates that *de novo* protein synthesis is required to cause disruption of calcium homeostasis in glutamate-treated cells. Mitochondrial proliferation and mitochondrial protein synthesis were suggested to occur during the course of neuronal apoptosis.[1] To evaluate the possible role of mitochondrial protein synthesis in the current glutamate-induced death pathway, we tested the effect of the mitochondrial ribosomal inhibitor chloramphenicol.[3] Chloramphenicol did not protect HT4 cells against glutamate-induced cytotoxicity, indicating that mitochondrial protein synthesis and biogenesis are not obligatory components of the death pathway. Taken together, these findings indicate that cellular *de novo* synthesis of proteins which are encoded by the nucleus and not by the mitochondrial genome are necessary to cause glutamate-induced cell death.

A key role of mitochondria in the death pathway was indicated by the evidence that the mitochondrial calcium uptake inhibitor Ruthenium Red[16] completely protected the cells against the glutamate cytotoxicity. Previously it has been reported for HT22 neuronal cells, a subclone of HT4,[11] that extracellular Ca^{2+} plays an important role in the excitotoxicity-independent cell death process of HT22 cells.[46] Our experiments with extracellular EGTA clearly indicated that extracellular Ca^{2+} does not contribute to the glutamate-induced increase in $[Ca^{2+}]_i$; in addition, pretreatment of the cells with 20 μM of $CoCl_2$ (an extracellular calcium influx blocker) did not protect the HT4 cells against glutamate cytotoxicity (data not shown). Thus, following glutamate challenge calcium that was mobilized from intracellular pools was sufficient to facilitate the HT4 cell death process. Accumulation of mitochondrial calcium has been shown to be an important component of neuronal death process.[24] Our findings support the hypothesis that mitochondrial Ca^{2+} uptake associated with mitochondrial degradation may be an important element in the execution of neuronal cell death.

Mitochondrial changes

Mitochondrial damage is known to play a major role in apoptotic processes.[9] Electron microscopic study of glutamate-treated cells showed mitochodrial degeneration, swelling and outer membrane damage. Mitochondrial swelling, spare cristae and decrease in optical density were reported previously to

occur during glutamate-induced cytotoxicity.[47] However, the issues of outer membrane integrity and the changes in membrane potential were not addressed in excitotoxicity-independent glutamate-induced cytotoxicity pathways.[47] Swelling was observed in the mitochondria of HT4 cells. Loss of MMP in response to glutamate treatment with a simultaneous increase in mitochondrial outer membrane permeability were observed, indicating a mechanism by which apoptogenic factors can be released from the mitochondria intermembrane space into the cytosol.

A significant part of the MMP was maintained during the first 8 h of glutamate treatment. Ca^{2+} accumulation into the mitochondria is known to be MMP dependent.[2,18] Calcium cycling (uptake and release) by the mitochondria has been shown to induce mitochondrial swelling and degradation eventually leading to cell death. The phenomenon of calcium cycling by mitochondria is known to be accelerated by oxidative damage and to be prevented by Ruthenium Red.[37,39] In the glutamate-treated cells Ruthenium Red treatment significantly lowered cytosolic $[Ca^{2+}]_i$, which may be due to a $[Ca^{2+}]_i$ buffering effect by other cellular organelles. Ruthenium Red protected the cells even when added 6 or 8 h following glutamate treatment and was able to recover the MMP. These findings indicate that mitochondrial degradation was a late event culminating in cell death. Mitochondrial swelling and loss of membrane potential are characteristics of MPT (pore opening). CsA did not protect the cells, suggesting a different mechanism for mitochondrial swelling or a CsA-insensitive form of MPT.

Partial maintenance of MMP during the early phase of the death process allows the mitochondria to continue accumulating cytosolic Ca^{2+}. Cycloheximide, Ruthenium Red and α-tocopherol normalized the MMP and prevented the increase in cyrosolic $[Ca^{2+}]_i$. This observation links $[Ca^{2+}]_i$ homeostasis to mitochondrial function. However, FCCP, a mitochondrial uncoupler, provided only partial protection to glutamate-treated cells. This minor protective effect may be due to slowing down of MMP-dependent Ca^{2+} uptake, as suggested previously in acute glutamate excitotoxicity where a combination of mitochondrial inhibitors (rotenone + oligomycin) collapsed the MMP and provided protection.[5]

In conclusion, mobilization of intracellular calcium and MMP-dependent calcium uptake by the mitochondria were noted to be major contributors to the cell death process. Mitochondrial dysfunction was found to be a key event in the excitotoxicity-independent component of glutamate-induced neuronal cell death. intracellular ROS accumulation and glutathione depletion were prominent in glutamate-treated cells but these events were not direct mediators of cell death.

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